



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Atty. Docket No:	001107.00363
Elizabeth Jaffee <i>et al.</i>	Confirmation No.	4098
Serial No.: 10/618,088	Group Art Unit:	1643
Filed: July 14, 2003	Examiner:	Bristol
For:	MESOTHELIN VACCINES AND MODEL SYSTEMS	

DECLARATION OF ELIZABETH M. JAFFEE

Commissioner of Patents
U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

I, Elizabeth M. Jaffee, M.D., hereby declare as follows:

1. I am a named inventor in the subject patent application and am familiar with its contents.
2. I am currently Co-Director of the Cancer Immunology Program and the Immunology and Hematopoiesis Division at Johns Hopkins Medicine and a Professor of Oncology, Immunology, and Pathology at The Johns Hopkins University School of Medicine. I have worked as a physician and researcher in the field of oncology for 18 years and, more specifically, have been involved in the field of cancer immunology for 17 years. I have authored numerous peer-reviewed articles in these fields and have made

a number of technical presentations at technical meetings in these fields. A copy of my curriculum vitae, including a list of my publications, is attached as Exhibit 1.

3. I am also a member of the Immunotherapy Scientific Advisory Board of Cerus Corporation, a licensee of the above-identified patent application.

Human studies with mesothelin-encoding tumor vaccine compositions:

4. I have been involved with or overseen a completed Phase I clinical trial (some of the results of which are reported in Examples 1-3 and Figures 1-6 in the subject patent application), two completed Phase II clinical trials, and an ongoing Phase II clinical trial for a vaccine composition comprising whole tumor cells that overexpress mesothelin in patients with surgically resected adenocarcinoma of the pancreas. The data from these clinical trials strongly support the efficacy of targeting mesothelin for pancreatic carcinoma in humans.

5. The vaccine composition used in the clinical trials was an allogeneic granulocyte-macrophage colony (GM-CSF)-secreting tumor cell vaccine composition. The tumor cell vaccine composition was produced by the stable transfection of two pancreatic adenocarcinoma cell lines derived from primary pancreatic tumor specimens (Jaffee et al., *Cancer J. Sci. Am.* 4:194-203 (1998)). Mesothelin is consistently and highly overexpressed in virtually all pancreatic adenocarcinomas at the mRNA and protein levels (Argani et al., *Clinical Cancer Research*, 7: 3862-3868 (2001)). Mesothelin is likewise encoded by and overexpressed in the GM-CSF-secreting tumor cell vaccine composition used in the Phase I and Phase II trials (Thomas et al., *Journal of Experimental Medicine*, 200: 297-306 (2004)).

6. Phase I trial: The data from the Phase I trial demonstrated that there is a direct correlation between observed post-vaccination induction of mesothelin-specific T cells, long term disease-free survival, and post-vaccination *in vivo* delayed-type hypersensitivity (DTH) responses to autologous tumor cells. These data are presented in Examples 1-3 and Figures 1-6 of the above-identified application and in Thomas et al.,

Journal of Experimental Medicine, 200: 297-306 (2004), a copy of which is attached as Exhibit 2. In addition, the mesothelin-specific T-cells that were induced were shown to lyse patient tumors *in vitro* (Thomas et al. (2004)).

7. In the Phase I clinical trial, the mesothelin-encoding tumor vaccine composition was administered to a total of 14 patients who had undergone surgically resection of adenocarcinoma of the pancreas and had minimal residual disease. Increased DTH responses to autologous tumor cells were induced in three of the 14 patients following vaccination with the tumor cell vaccine, and the responses were found to correlate with an increased disease-free survival time for these three patients. (Jaffee et al., *Journal of Clinical Oncology*, 19: 145-156 (2001)). To date, the three DTH responders have now lived over eight years from the time of diagnosis with a disease for which the median survival time for patients (with operable cancer) is only approximately 17 to 22 months. The early data from the Phase I trial indicated that the tumor cell vaccine induced a dose-dependent systemic anti-tumor immunity in the three long-term survivors (Jaffee et al., *Journal of Clinical Oncology*, 19: 145-156 (2001)).

8. We found that the post-vaccination induction of mesothelin-specific T-cells correlated with long term survival in the DTH responders. These results are reported in Examples 1-3 and Figures 1-6 of the subject patent application and in Thomas et al. (2004). We discovered that vaccination with the tumor vaccine had resulted in the consistent induction of CD8⁺ T cell responses to multiple HLA-A2, A3, and/or A24-restricted mesothelin epitopes (SEQ ID NOS: 1-6 in the above-identified patent application) in the three patients in the Phase I study with vaccine-induced DTH responses. By contrast, only one of eleven DTH non-responders had an increased post-vaccination mesothelin-specific T cell response and, even then, only to a single peptide. The CD8⁺ T cell response was determined by screening antigen-pulsed, TAP transporter deficient, T2 cells with CD8⁺ T cell enriched peripheral blood lymphocytes (PBLs) obtained pre- and post-vaccination from the patients in a quantitative ELISPOT assay.

10. The mesothelin-specific T cells from one of the DTH responders were also shown to be capable of lysing mesothelin-expressing cell lines. As reported in Thomas et al. (2004), we analyzed the reactivity of a patient-derived T-cell line to a panel of mesothelin-expressing tumor cell lines in a 4-h chromium release assay. After *in vitro* stimulation of the patient-derived T cells, the T-cells were shown to be capable of lysing mesothelin-expressing cells, including a mesothelin-expressing pancreatic tumor cell line. These data confirm that the mesothelin-encoding tumor cell vaccine composition can induce mesothelin-specific T-cells that can be cytolytic.

11. The results of the quantitative ELISPOT assays indicate that mesothelin epitopes provided by the mesothelin encoded by the administered vaccine composition bound to MHC class I molecules and were successfully processed and presented by professional antigen presenting cells (APCs) in each of the DTH responders. The CD8⁺ T cell activation in the DTH responders must have occurred by transfer of the MHC class I antigens from the tumor cell vaccine to professional antigen presenting cells, where they must have been properly processed and presented on MHC class I molecules via cross-priming. This is evidenced by the fact that while the cells lines used to produce the whole tumor cell vaccine overexpressed mesothelin, neither tumor cell line expressed HLA-A2, A3, or A24 and thus the vaccine cells were mismatched at the HLA-A locus with all three DTH responders (Thomas et al. (2004)).

12. Phase II trials: A Phase II study in metastatic cancer patients was subsequently performed and confirmed the results of the Phase I trial. Patients received either the tumor cell vaccine composition alone (30 patients) or the tumor cell vaccine composition plus immune-modulating doses of cyclophosphamide (20 patients) to remove inhibitory regulatory T cells prior to vaccination. Mesothelin-specific T cells were induced in about a third of the patients treated in this trial. Those patients who demonstrated mesothelin responses also demonstrated prolonged, progression-free survival. The patient with the most durable response went on to disease-free survival for greater than 2 years.

13. In addition, the interim results of a 60-patient Phase II trial further confirm those of the Phase I trial which indicated that mesothelin is effective as an immune target for pancreatic carcinoma. The treatment phase for the Phase II 60-patient study with the mesothelin-encoding tumor cell vaccine composition for patients who were surgical candidates has just been completed. We reported the early analysis at the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, held November 14 -18, 2005, in Philadelphia, Pennsylvania, and the American Society of Clinical Oncology (ASCO) 2007 Gastrointestinal Cancers Symposium, held January 19-21, 2007, in Orlando, Florida. Copies of abstracts for these presentations are attached as Exhibit 3. Our one and two year survival data are 88% and 76% respectively, which is considered better than what has previously been reported for other adjuvant therapies.

14. So far, 16 of the 60 patients in the most recent Phase II trial have been analyzed for the induction of mesothelin responses. All 16 have demonstrated some level of mesothelin response over the course of multiple treatments with the vaccine. These responses were correlated with changes in CA19.9 levels, which are a measure of cancer burden (see Exhibit 4). Twelve of the 16 patients demonstrated a direct correlation strongly suggesting that mesothelin-specific T cell responses increase and remain elevated when CA19.9 is low and decrease when CA19.9 increases (tumor recurrence).

In vivo mouse model studies with mesothelin-encoding DNA vaccine compositions:

15. In addition to the human studies described above, I am familiar with *in vivo* mouse model studies that shows the efficacy of mesothelin-encoding DNA vaccine compositions. The results of these studies are described in Examples 5-12 and Figures 7-12. These studies further validate the efficacy of mesothelin as an immune target in mammals for the treatment of tumors, such as pancreatic tumors, that overexpress mesothelin.

16. The *in vivo* studies described in Examples 5-12 of the subject application utilize an ascitogenic WF-3 tumor cell line. Examples 5-10 support the suitability of the WF-3 tumor cell line as an *in vivo* model for the testing of immunotherapeutics against

mesothelin-expressing tumors such as ovarian cancer and pancreatic cancer. Examples 5-10 of the application describe the construction and characterization of the WF-3 tumor cell line and its use in an *in vivo* mouse model. The WF-3 tumor cell line was shown to be positive for MHC class I expression (Example 7 and Figure 8). Mesothelin was shown to be highly expressed in the WF-3 cells (Examples 9 and 10).

17. The WF-3 tumor cell model is a particularly suitable model for assessing the effects of candidate cancer vaccines targeting tumors which overexpress mesothelin, including both ovarian cancer and pancreatic cancer, because WF-3 cells endogenously express mesothelin. Many models use tumors that are genetically modified to express an exogenous tumor antigen. In tumors that are genetically modified to express an exogenous antigen, the antigen may be presented in abundance and may be easier to recognize by T-cells due to the large number of antigen/major histocompatibility complex molecules available. In addition, a genetically modified tumor antigen likely cannot be down-regulated by natural mechanisms in the tumor micro-environment due to immune suppression. In contrast, the WF-3 model is more similar to native tumors because it presents mesothelin at natural levels and is subject to natural tolerance mechanisms. In addition, the WF-3 cells grow in immunocompetent mice, which permits the investigation of immune mechanisms not permitted in nude or SCID immunocompromised mice. Moreover, the WF-3 cells form ascites in the peritoneum; one of the primary sites for pancreatic cancer metastases is the peritoneum.

18. Example 11 of the subject application reports the generation of a DNA vaccine ("pcDNA3-mesothelin") that encodes murine full-length mesothelin protein and administration of the vaccine to a mouse. The DNA vaccine was constructed from a mammalian cell expression vector. A gene gun was used to deliver gold particles coated with the mesothelin-encoding DNA to C57BL/6 mice and to administer a booster to the mice a week later. A week later, the mice were challenged with a lethal dose of WF-3 tumor cells.

19. In the *in vivo* mouse model experiments described in Example 11, the mesothelin-encoding DNA vaccine composition was shown to provide a high degree of protection (60%) against lethality caused by the WF-3 tumor cell challenge compared to the "vector only" and "no vaccination" controls (Example 11 and Figure 11). In addition, the DNA vaccine was shown to be capable of inducing mesothelin-specific T cell-mediated specific lysis of WF-3 cells (Example 12 and Figure 12). The data in Examples 11 and 12 and Figures 11 and 12 indicate that a mesothelin-encoding DNA vaccine composition can generate mesothelin-specific, antitumor T-cell immune responses in a mammal and can be used in the treatment or control of cancers in which mesothelin is highly expressed.

In vivo mouse model studies with mesothelin-encoding Listeria-based vaccine compositions:

20. In addition to the above-referenced whole tumor cell vaccine trials in humans and DNA vaccine studies in the WF-3 mouse model, I am also aware of and familiar with *in vivo* mouse model studies involving mesothelin-encoding, *Listeria*-based vaccine compositions that express polypeptides comprising mesothelin epitopes. These studies provide further support for the efficacy of mesothelin-encoding vaccine compositions, the ability of mesothelin-encoding vaccine compositions to induce anti-tumor T-cell responses, and the efficacy of mesothelin as an immunotherapeutic target in cancers such as pancreatic cancer in which mesothelin is highly expressed.

21. U.S. Patent Publication No. 2005/0249748: U.S. Patent Publication No. 2005/0249748 reports several studies demonstrating the effects of vaccination of mice with *Listeria* that express fusion proteins comprising either human mesothelin or human mesothelin deleted of its signal sequence and GPI anchor operably linked to a heterologous signal sequence (Exhibit 5).

22. Example 31B of U.S. Patent Publication No. 2005/0249748 (paragraphs [0603] – [0606] and Figure 61) reports that CT26 murine colon carcinoma tumor cells transduced to express human mesothelin were injected intravenously into BALB/c mice to form tumors. Attenuated *Listeria monocytogenes* that had been genetically modified to

express and secrete human mesothelin deleted of its signal sequence and GPI anchor was administered as a vaccine to the tumor-bearing BALB/c mice. (The mesothelin sequence deleted of its signal sequence and GPI anchor comprises epitopes of SEQ ID NOS: 2-5 listed in the subject application.) As shown in Figure 61, a single immunization with *Listeria*-based vaccine composition encoding a mesothelin sequence ("BaPA-huMeso Δ gpi Δ ss") prolonged survival of the mice containing the mesothelin-expressing tumors relative to the negative controls comprising no mesothelin coding sequences ("HBSS" and "actAinIB") ("SF-AH1A5" represents a positive but unrelated anti-tumor control.)

23. Example 31C of U.S. Patent Publication No. 2005/0249748 (paragraphs [0608] – [0611] and Figure 62) indicates that the level of lung tumor nodules *in vivo* is reduced by vaccination with either of two different mesothelin-encoding *Listeria*-based vaccine compositions. Attenuated *Listeria* comprising a sequence integrated in its chromosomal DNA that encodes a polypeptide comprising human mesothelin deleted of its signal sequence and GPI anchor ("BaPA-HuMeso Δ gpi Δ ss"), attenuated *Listeria* comprising plasmid DNA encoding a polypeptide comprising human mesothelin ("pAM-LLO-HuMeso"), or controls were administered to BALB/c mice after the mesothelin-expressing CT26 murine colon carcinoma tumor cells were injected intravenously into the mice. The mice were eventually sacrificed and the lung tumor nodules counted. The results shown in Figure 62 indicate that the number of lung tumor nodules was reduced significantly when either of the two *Listeria*-based vaccine compositions encoding mesothelin sequences were administered, relative to the negative control (*i.e.*, *Listeria* which does not encode mesothelin). Furthermore, the anti-tumor efficacy of vaccination with the *Listeria* vaccine encoding mesothelin was shown to be mesothelin-specific (paragraphs [0610]-[0611] and Figure 63).

24. Example 31D of U.S. Patent Publication No. 2005/0249748 (paragraphs [0612] – [0618] and Figure 64) demonstrates that vaccination with a mesothelin-encoding *Listeria*-based vaccine reduces tumor volume in an *in vivo* model. BALB/c mice were implanted subcutaneously with the CT26 tumor cells and later vaccinated with one of three different mesothelin-encoding *Listeria*-based vaccine compositions or a positive or negative

control. The mesothelin-encoding *Listeria*-based vaccine compositions comprised either a plasmid encoding a fusion protein comprising human mesothelin ("pAM opt.LLO-opt.huMeso" or "pAM non-opt.BaPA-opt.huMeso") or a chromosomally-integrated sequence encoding a fusion protein comprising human mesothelin deleted of its signal sequence and GPI anchor ("Non-opt.BaPA-opt.huMeso delgpi-ss"). The results shown in Figure 64 indicate that administration of any one of the three vaccine compositions encoding mesothelin sequences resulted in a decreased tumor volume relative to the negative control (*i.e.*, "HESS").

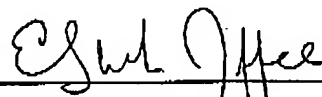
25. SPORE 2006 poster: I am also aware of additional experiments involving *Listeria*-based vaccine compositions that were the subject of a poster by Brockstedt et al., entitled "CRS-207: Live-Attenuated *Listeria monocytogenes* Encoding Mesothelin for Immunotherapy of patients with Pancreatic and Ovarian Cancers," and presented at the 14th Annual SPORE Investigators' Workshop, July 16-19, 2006, in Baltimore, Maryland. A copy of the poster presentation is attached as Exhibit 6. I am named as a co-author on the poster.

26. The 2006 SPORE poster reports studies which demonstrate that the level of lung tumor nodules *in vivo* is reduced by vaccination with a mesothelin-encoding *Listeria*-based vaccine composition (Figure 5). Recombinant, attenuated *Listeria monocytogenes* comprising a sequence integrated into its chromosomal DNA encoding a polypeptide comprising human mesothelin deleted of its signal sequence ("CRS-207") or negative or positive controls were administered to BALB/c mice after mesothelin-expressing CT26 murine colon carcinoma cells were injected intravenously into the mice. (The mesothelin sequence deleted of its signal sequence retains the epitopes of SEQ ID NOS: 2-5 listed in the above-identified application.) The mice were eventually sacrificed and the lung tumor nodules counted. The pictures of the lungs shown in Figure 5 clearly show that vaccination with the mesothelin-encoding vaccine composition led to a reduction in tumor nodules.

27. Also the 2006 SPORE poster reports studies which demonstrate that the anti-tumor efficacy of the mesothelin-encoding *Listeria*-based vaccine compositions is due to innate and mesothelin-specific cellular immunity (Figure 6). In these experiments, NK cells, CD4⁺ T cells, or CD8⁺ T cells were depleted by antibodies in some mice prior to tumor cell implantation. The mice were then vaccinated with the same mesothelin-encoding *Listeria*-based vaccine composition as was used in the experiments shown in Figure 5 ("CRS-207"). The survival data are shown in Figure 6 and indicate that vaccination with the mesothelin-encoding vaccine composition leads to enhanced survival. The data also show that the depletion of CD8⁺ T cells, CD4⁺ T cells, or NK cells impairs the anti-tumor immunity.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

4-27-07
Date


Elizabeth Jaffee, MD

Exhibits:

1. *Curriculum Vitae*
2. Thomas et al. *Journal of Experimental Medicine*, 200: 297-306 (2004)
3. AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, held November 14 -18, 2005, in Philadelphia, Pennsylvania, and the American Society of Clinical Oncology (ASCO) 2007 Gastrointestinal Cancers Symposium
4. Boeck S, *Onkologie* 2007 abstract; Locker GY, et al, *J. Clin. Oncol* 2006 abstract
5. U.S. Patent Publication No. 2005/0249748

6. Brockstedt et al., entitled "CRS-207: Live-Attenuated *Listeria monocytogenes* Encoding Mcsorthelin for Immunotherapy of patients with Pancreatic and Ovarian Cancers," and presented at the 14th Annual SPORE Investigators' Workshop, July 16-19, 2006

EXHIBIT 1

CURRICULUM VITAE

Elizabeth M. Jaffee

December 18, 2006

DEMOGRAPHIC INFORMATION:

Current Appointments:

University: Professor of Oncology
The Johns Hopkins University School of Medicine

Professor of Pathology
The Johns Hopkins University School of Medicine

Hospital: Active Staff in Oncology
The Johns Hopkins Hospital

Personal Data:

Addresses:

Office: Department of Oncology
The Sidney Kimmel Comprehensive Cancer Center
At Johns Hopkins
1650 Orleans Street
Bunting-Blaustein Building Room 4M07
Baltimore, MD 21231
Telephone: (410) 955-2957
Fax: (410) 614-8216
e-mail: ejaffee@jhmi.edu

Educational and Training:

1977 - 1981 B.A. 1981 Magna cum laude
Brandeis University
Waltham, Massachusetts

1981 - 1985 M.D., New York Medical College
Valhalla, New York

1985 - 1988 Intern and Resident
Department of Medicine
University of Pittsburgh
Presbyterian-University Hospital
Pittsburgh, Pennsylvania
Gerald S. Levey, M.D., Chairman

1988 - 1989 Research Fellow, Physician Investigator
NIH Research Training Grant #5T32DKO745807
Department of Medicine

The University of Pittsburgh
Pittsburgh, Pennsylvania
Fran Finn, Ph.D., Research Director

1989 - 1992 Senior Clinical/Research Fellow in Oncology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

1989-1992 Senior Clinical Fellow in Oncology
The Johns Hopkins Hospital
Baltimore, Maryland

Professional Experience:

1992 - 1997 Assistant Professor of Oncology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

1992 - present Medical Director, Johns Hopkins Oncology Center
Cell Processing and Gene Therapy Facility

1995-present Faculty, Graduate Program in Immunology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

1997-2002 Associate Professor of Oncology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

2002-present Professor of Oncology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

1999-present Faculty, Graduate Program in Cellular and Molecular
Medicine
The Johns Hopkins University School of Medicine
Baltimore, Maryland

1999-2002 Associate Professor of Pathology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

2002-Present Professor of Pathology
The Johns Hopkins University School of Medicine
Baltimore, Maryland

2000-present Faculty, Graduate Program in Pharmacology
The Johns Hopkins University School of Medicine

2004-2006 Chairperson, clinical Research committee

RESEARCH ACTIVITIES

Peer-Reviewed Publications

1. Golumbek PT, Lazenby AJ, Levitsky HI, **Jaffee EM**, Baker M, Pardoll DM. Treatment of established renal cancer by tumor cells engineered to secrete IL-4. *Science*; 251:713-715, 1991.
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11. **Jaffee EM** and Pardoll DM. Gene Therapy: Its Potential Application in the Treatment of Renal Cell Carcinoma. *Semin. Oncol.*; vol 22 (1), 81-91, 1994.

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14. Griffin CA, Hruban RH, Morsberger LA, Ellingham T, Long PP, **Jaffee EM**, Hauda KM, Bohlander K, Yeo CJ. Consistent chromosome abnormalities in adenocarcinoma of the pancreas. *Cancer Research* 55:2394-2399, 1995.
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18. Thompson RC, Pardoll DM, **Jaffee EM**, Ewend MG, Thomas M, Tyler B, Brem H. Systemic and local paracrine cytokine therapies using transduced tumor cells are synergistic in treating intracranial tumors. *J Immunotherapy*, 19(6):405-413, 1996.
19. Huang AYC, Gulden PH, Woods AS, Thomas MC, Tong CD, Wang W, Engelhard VH, Pasternack G, Cotter R, Hunt D, Pardoll DM, **Jaffee EM**. The Immunodominant MHC Class I-Restricted Antigen of a Murine Colon Tumor Derives From an Endogenous Retroviral Gene Product. *PNAS* 93:9730-9735, 1996.
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- gene-transduced tumor cell vaccines. *Methods* 12(2):143-153, 1997.
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58. Emens LA, **Jaffee EM**. Cancer vaccines: an old idea comes of age. *Cancer Biol Ther.* 2(4 Suppl 1):S161-8; 2003.
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61. Ercolini AM, Machiels J-P, Chen Y-C, Slansky JE, Giedlen M, Reilly RT, **Jaffee EM**: Identification and Characterization of the Immunodominant Rat HER-2/neu MHC Class I Epitope Presented by Spontaneous Mammary Tumors from HER-2/neu-Transgenic Mice. *J. Immunol.*, 170: 4273-4280; 2003.
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66. Gandini S, Lowenfels AB, **Jaffee EM**, Armstrong TD, Maisonneuve P. Allergies and the Risk of Pancreas Cancer: A systematic Review and Meta-Analysis. Submitted 2004.
67. Mautner J, Deckhut A, **Jaffee EM**, Pardoll DM. Tumor-specific CD4+ cells from a patient with renal cell carcinoma recognize diverse shared antigens. *International Journal of Cancer*. 2005, Feb 9.
68. Zhou X, Jun DY, Thomas AM, Huang X, Huang L-Q, Mautner J, Yu Zhiwei, Mo W, Robbins PF, Pardoll DM, **Jaffee EM**. Diverse CD8+ T Cell Responses to Renal Cell Carcinoma Antigen in a Patient Treated with an Autologous GM-CSF Gene-Transduced Renal Tumor Cell Vaccine. *Cancer Research* 65(3):1079-88, 2005.
69. Ercolini AM, Ladle BH, Manning EA, Pfannenstiel LW, Armstrong TD, Machiels J-P, Bieler JG, Emens LA, Reilly RT, **Jaffee EM**. Recruitment of latent pools of high avidity CD8+ T cells to the antitumor immune response. *Journal of Experimental Medicine*, 201 (10):1591-1602, 2005
70. Davis-Sproul JM, Harris MP, NE Davidson, Kobrin BJ, **Jaffee EM**, Emens LA. Cost-effective manufacture of an allogeneic GM-CSF-secreting breast tumor vaccine in an academic cGMP facility. *Cytotherapy* 7(1): 46-56, 2005.
71. Gandini S, Lowenfels A, **Jaffee EM**, Armstrong T, Maisonneuve P. Allergies and the Risk of Pancreatic Cancer: A Meta-analysis with Review of epidemiology and Biological Mechanisms. *Cancer Epidemiol Biomarkers Prev* 14(8), August 2005.
72. Singh R, Dominiacki ME, **Jaffee EM**, Paterson Y. Fusion to Listeriolysin O and delivery by *Listeria monocytogenes* enhances the immunogenicity of HER-2/neu and reveals subdominant epitopes in the FVB/N mouse. *J Immunol*. 175(6): 3663-73, Sep 2005.
73. Murata S, Ladle BH, Kim PS, Lutz ER, Wolpoe ME, Smith HM, Armstrong TA, Emens LA, **Jaffee EM**, Reilly RT. OX40 Costimulation Synergizes with GM-CSF Whole-Cell Vaccination to Overcome Established CD8+ T Cell Tolerance to an Endogenous Tumor Antigen. *J. Immunol*. 176(2): 974-83, Jan 2006.
74. Murata S, Kominsky SL, Vali M, Zhang Z, Garrett-Mayer E, Korz D, Huso D, Baker SD, Barber J, **Jaffee E**, Reilly RT, Sukumar S. Ductal access for prevention and therapy of mammary tumors. *Cancer Res*. 66(2): 638-45, Jan 2006.
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Invited Chapters and Reviews.

1. **Jaffee EM** and Pardoll DM. (1994) Cytokine Gene-Transduced Tumor Cell Vaccines in "The Handbook of Experimental Immunology.". (Eds: DM Weir, C Blackwell,

Leonard A Herzenberg, and Leonore A Herzenberg). Blackwell Scientific Publications, Oxford, 5th Edition.

2. **Jaffee EM.** (1994) Methods for Retroviral-Mediated Gene Transfer to Tumor Cells in "Methods in Molecular Biology: Gene Therapy Protocols." (Ed. Paul Robbins). The Humana Press, Inc., New Jersey, 307-327.
3. **Jaffee EM, Hurwitz, H and Pardoll D** (1995) Immunotherapy with Genetically Modified Tumor Vaccines in "Biologic Therapy of Cancer." (Eds. DeVita VT, Hellman S, and S Rosenberg), Lippincott Press, Philadelphia, 2nd Edition, 774-783.
4. **Jaffee EM, Pardoll DM.** (1996). Cytokine Gene-Transduced Tumor Vaccines in "Therapeutic Immunology". (Eds. K. Frank Austen, Steven J. Burakoff, Fred S. Rosen, Terry B. Strom). Blackwell Science, Inc., Cambridge, Massachusetts, 1st Edition, 575-586.
5. **Jaffee EM, Pardoll DM.** (1996) Murine Tumor Antigens: Is it Worth the Search? Current Opinion Immunology 8:622-627.
6. Greten T, **Jaffee EM.** (1996) Methods for Gene Transfer to Primary Human Tumors. Current Protocols in Human Genetics. (Eds: N. C. Dracopoli, J.L. Haines, B.R. Korf, D.T. Moir, C.C. Morton, C. E. Seidman, J.G. Seidman, D.R. Smith). John Wiley & Sons, Inc., New York, New York.
7. **Jaffee EM** (1999) Cancer Immunotherapy Protocols in "Methods in Molecular Medicine".
8. **Jaffee, E.M.** (2000) Immunotherapy of Cancer. Anticancer Molecules. Annals of the New York Academy of Sciences 886,:67-72.
9. Reilly RT, Machiels JP, **Jaffee EM.** (2000) Cancer Vaccines. Emerging drugs 5(2):201- 209.
10. Marincola FM, **Jaffee EM, Hicklin DJ, Ferrone S.** (2000) Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv Immunol. 74:181-273
11. Laheru D, Biedrzycki B, **Jaffee EM.** (2001) Immunologic Approaches to the Management of Pancreatic Cancer. The Cancer Journal 7(4):324-327.
12. Reilly RT, Machiels JP, Emens LA, **Jaffee EM.** (2001) Cytokine Gene-Modified Cell-Based Cancer Vaccines, "Methods in Molecular Medicine", Vol. 69, Gene Therapy Protocols, 2nd Ed. Cytokine Gene-Modified Cell-Based Cancer Vaccines, Humana Press Inc., New Jersey, 233-257.
13. **Jaffee EM, Hruban RH, Canto M, Kern SE.** (2002) Focus on pancreas cancer. Cancer Cel., 225-282.

14. Emens, LA and **Jaffee, EM.** (2003) Gene-Modified Tumor Cell Vaccines. In: *Handbook of Cancer Vaccines*, Humana Press, ed. Morse, MA, Clay, TM, and Lysterly, HK, pp253-273.
15. Emens, LA and **Jaffee, EM.** (2003) Cancer Vaccines: An Old Idea Comes of Age. *Cancer Biology and Therapy* 2(4 Suppl 1):S161-8.
16. Laheru D, Biedrzycki B, Thomas AM, **Jaffee EM.** (2003) Development of a Cytokine-modified Allogeneic Whole Cell Pancreatic Cancer Vaccine. *Methods in Molecular Medicine*, 103:299-328.
17. Emens LA, Reilly RT, **Jaffee EM.** (2004) Augmenting the Potency of Breast Cancer Vaccines: Combined Modality Immunotherapy. *Breast Disease* 20: 13-24.
18. Emens, LA, Reilly RT, and **Jaffee, EM.** (2005) Manipulating Immunologic Checkpoints to Maximize Antitumor Immunity. In: *Immunotherapy of Cancer* ed. Nora Disis, Humana Press, pp. 331-353.
19. Emens, LA and **Jaffee, EM.** (2005) Leveraging the Activity of Therapeutic Cancer Vaccines with Cytotoxic Chemotherapy. *Cancer Research*, 65: 1-6.
20. Emens, LA and **Jaffee, EM.** (2005) Leveraging the Activity of Tumor Vaccines with Cytotoxic Chemotherapy. *Cancer Research*, 65: 18
21. Emens, LA, Reilly RT, **Jaffee, EM.** (2005) Cancer Vaccines in Combination with Multimodality Therapy. *Cancer Treat Research*, 123: 227-45.
22. Laheru DA, Pardoll DM, **Jaffee, EM.** (2005) Genes to Vaccines for Immunotherapy: How the Molecular Biology Revolution has Influenced Cancer Immunology. *Molecular Cancer Therapeutics*, 4(11): 1645-52.
23. Emens LA, Reilly RT, Jaffee EM. (2005) Breast Cancer vaccines: maximizing cancer treatment by tapping into host immunity. *Endocrine-Related Cancer*, 12(1):1-17.
24. Laheru D, **Jaffee EM.** (2005) Pancreas Cancer Immunotherapy: Science Driving Clinical Progress. *Nature Reviews Cancer*, 5(6): 459-467.
26. Drake CG, **Jaffee, EM,** Pardoll DM. (2006) Mechanism of Immune Evasion by Tumors. *Advances in Immunology*, 90: 51-81.

Patents:

1. Methods of Treating Cancer with a Tumor Cell Line Having Modified Cytosine Expression.. Inventors: Elizabeth M. Jaffee, Drew M. Pardoll, and Hyam I. Levitsky. U.S. Patent# 6,033,674.

2. Allogeneic Paracrine Cytosine Tumor Vaccines. Elizabeth M. Jaffee, Drew M. Pardoll, and Hyam I. Levitsky. U.S. Patent# 8,773,367.
3. Controlled Release of Pharmaceutically Active Substances for Immunotherapy. Inventors: Drew M. Pardoll, Elizabeth M. Jaffee, Paul Golumbek, Hyam Levitsky, and Kam Leong. U.S. Patent #5,861,159.
4. Cytokine Enhanced Immunotherapy for Brain Tumors. Inventors: Henry Brem, Drew Pardoll, Elizabeth Jaffee, Kam Leong, Reid Thompson. Pending.
5. Melanoma Cell Lines Expressing Shared Immunodominant Melanoma Antigens and Methods of Using Same. Drew M. Pardoll, Elizabeth M. Jaffee, Adam Adler, Suzanne L. Topalian, and Steven A. Rosenberg. U.S. Patent #6,187,306.
6. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. Inventors: Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, Laheru DA, Goggins M, Hruban RH, Jaffee EM. U.S. Patent pending.

Extramural Sponsorship:

Grants:

Previous:

Principal Investigator, NIH/NCI K11 Award, "Isolation and Characterization of Tumor-Specific Antigens", Project Dates: 08/01/92 - 07/31/97. Total costs: \$716,027

Principal Investigator, ACS Career Development Award, "Isolation and Characterization of Tumor-Specific Antigens", Project Dates: 07/01/92 - 06/30/93. Total Costs: \$28,000

Principal Investigator, ASCO Award, "Isolation and Characterization of Tumor-Specific Antigens", Project Dates: 07/01/92 - 06/30/93. Award Total: \$26,500

Principal Investigator, NIH/NCI CORE Grant Awards, "Development of a Breast Cancer Program at Hopkins", Project Dates; 09/3-/94 - 09/29/95. Award Total: \$33,375

Principal Investigator, National Kidney Cancer Assoc., "Identification of Human Renal Cancer Antigens Recognized by T Cells", Project Dates: 07/01/95 - 06/30/96. Award Total: \$30,000

Principal Investigator, NIH/NCI 1R01CA95012, "A phase I trial of an allogeneic pancreatic cancer vaccine", Project Dates: 07/01/96 - 06/30/00, Award Total: \$609,503.00

Principal Investigator, Army - Department of Defense DAMD17-96-1-6138
“Recombinant vaccine strategies for breast cancer prevention”, Project Dates:
10/01/96 - 09/30/00, Award Total: \$685,000.

Project Leader and Co-Investigator, NIH - NCDDG CA-95-020, “Antigen specific
vaccines for breast and cervical cancer”, Principal Investigator: Pardoll, Project
Dates: 11/1/96 - 9/30/00, Award Total: \$723,584

Project Leader, DOD DAMD17-01-1-0280, “Identification of widely applicable
tumor associated antigens for breast cancer immunology”, Principal Investigator: Bai,
Project Dates: 09/15/01 -09/14/04, Award Total: \$100, 000 (annual).

Principal Investigator, NIH/NCI 1R01CA79685 “Identification of CD8+ T cell
targets on renal cancer”, Project Dates: 12/1/99 – 11/30/04, Award Total: \$1,374,359

Principal Investigator, NIH/NCI NCDDG 2U19CA72108, “Antigen-specific vaccines
for breast cancer”, Project Dates: 09/19/00 - 04/30/05, Award Total: \$1,414,600

Principal Investigator, NIH/NCI 1 R01 CA88058, “A phase II clinical trial testing the
efficacy of a GM-CSF secreting allogeneic pancreatic tumor vaccine for the treatment
of pancreatic adenocarcinoma”, Project Dates: 07/01/00 - 12/30/05, Award Total:
\$ 1,599,665

Current:

Principal Investigator, NCDDG NIH/NCI, “Combinatorial immunotherapy to amplify
vaccine induced immunity”, Project Dates: 6/1/05 – 04/30/10, Award Total:
\$733,945/year.

Project Leader and Co-Principal Investigator, 1P50CA88843, SPORE in Breast
Cancer, “Vaccines: A New Paradigm for Breast Cancer Prevention”, Co-Principal
Investigator: Nancy Davidson, Project Dates: 09/30/00 – 09/29/06, Award Total:
\$1,429,350

Project Leader, NIH-RFA P50 CA62924, “SPORE in Gastrointestinal Cancer”,
“Gastrointestinal Malignancies: Integration of Chemotherapy with Vaccination in
Metastatic Pancreatic Cancer” Principal Investigator: Scott Kern, Project Dates:
10/01/93 - 06/30/07, Award Total: \$863,298

Project Leader, DOD/COE, “Prevention and Therapy of Metastatic Breast Cancer”,
high avidity HER-2/neu specific T cells traffic and function at the site of metastases.
There is no overlap. Principal Investigator: Saraswati Sukumar, Project Dates: 7/1/04
– 06/30/09, Award Total: \$200,000/year.

Principal Investigator, NIH/NCI RO1 CA93714, “Chemotherapy plus vaccine for
metastatic breast cancer”: Project Dates: 01/01/02 – 12/31/07, Award Total:
\$2,984,128.

Principal Investigator, Avon Foundation, "The Avon Baltimore/Seattle Breast Cancer Immunotherapy Collaborative" Project Dates: 10/10/02 – 09/30/08 Total Award: \$2,680,000.

Contracts:

Previous:

Principal Investigator, SmithKline Beecham, "Identification of genes encoding the MHC class I and II antigens expressed by human renal, prostate and renal carcinoma cells", Project Dates: 04/30/96 - 03/14/98. Award Total: \$390,125.27

Principal Investigator, SmithKline Beecham, "Phase I and II an allogeneic pancreatic clinical trial" Project Dates: 2/29/96 - 2/28/00, Award Total: \$1,035,150.0

Principal Investigator: Corixa, "Isolation of pancreatic tumor antigens", Project Dates: 01/01/01 – 12/31/01, Award Total: 141,346

Principal Investigator, Lustgarten Foundation, "A phase II study of an allogeneic GM-CSF secreting pancreatic tumor vaccine", Project Dates: 10/1/99 – 9/30/03 Award Total : \$700,000

Principal Investigator, Cell Genesys, "Development of vaccine strategies potent enough to activate low affinity T cells", Project Dates: 06/01/00 - 11/30/03, Award Total: \$514,872

Principal Investigator, Cell Genesys, "A phase II trial of CG8020 and CG2505 in patients with nonresectable or metastatic pancreatic cancer", Project Dates: 07/01/02 - 06/30/04, Award Total: \$346,908

Current:

Principal Investigator, Cell Genesys, "A safety and efficacy trial of lethally irradiated allogeneic pancreatic tumor cells transfected with the GM-CSF gene in combination with adjuvant chemoradiotherapy for the treatment of adenocarcinoma of the pancreas", Project Dates: 10/01/01 - 09/30/06, Award Total: \$848, 000

Principal Investigator, Cerus, "Evaluation of mesothelin as a vaccine target" Developing new mesothelin based vaccines in a new mouse model of mesothelin expressing pancreatic tumors. Project dates 8/1/04 - 7/31/06, Award Total: \$116,883/year.

EDUCATIONAL ACTIVITIES:

Teaching:

1992 - present Second Year Medical Student Neoplastic Diseases
Pathophysiology Course – Coordinator, Lecturer

1994 – present Precept Clinical Oncology Fellows in weekly outpatient clinic

1995 - present	First Year Medical Student Immunology Course - Lecturer and small group section leader
1995 – 2003.	Organizer of Weekly Immunology Research Conference in Immunology Program
1995 - present	Faculty, Graduate Program in Immunology
1997 – 2001.	Clinical Faculty Instructor, Introductory to Pathobiology - Lecturer, Pathobiology of Cancer Training Program
1997 – present	Director, Second Year Medical Student Neoplastic Diseases Pathophysiology Course
1998- 2003	Lecturer in the JHU undergraduate immunology course – Tumor Immunology
1998 - present	Lecturer in the JHU School of Public Health immunology course – Tumor Immunology.
1999 - present	Faculty, Graduate Program in Cellular and Molecular Medicine
2000 - present	Faculty, Graduate Program in Pharmacology
2001 – 2003.	Tutorial in Pharmacology: Principles and the Clinical Application of Genetic Target Discovery
2006 – present	Cell and Molecular Medicine

Mentoring Advisees:

Post-doctoral Fellows

1. Todd Armstrong. 9/02-7/04. Development of TCR transgenic mice specific for the immunodominant HER-2/neu CD8+ T cell antigen. Current position. Research Associate. Johns Hopkins School of Medicine.
2. Yi-Cheng Chen, M.D, 3/01 – 2003. Assessment of immune responses to DC based vaccines. Assistant Professor, Taiwan.
3. Alison Deckhut, Ph.D. Assessment and Identification of the Antigens expressed by human renal tumors. 11/94 - 5/97. Current position: Faculty Member, NIH
4. Kathy Dixon, Ph.D. Antigen based strategies for the treatment of breast cancer. 11/94 - 2/96. Current Position: Senior Researcher, Genetic Therapy Inc.
5. Leisha Emens, M.D. 6/99 - present. Assistant Professor of Oncology, Johns Hopkins University SOM, November 1, 2001. Received a high priority score on a K23 NIH award that was funded; with Dr. Jaffee as her mentor.

6. Priyadarshini Ganesan, Ph.D.. August 2004-February 2006. Analysis of immune tolerance mechanisms to a new tumor antigen, mesothelin, in a murine pancreatic tumor model.
Current Position: Research Associate, Australia.
7. Tim Greten, M.D. Evaluation of Tumor antigens expressed by pancreatic tumors. 9/95 - 10/98. Current Position: Faculty, MH-Hanover, Germany
8. Lan-Qing Huang, M.D. 8/00 – 7/03. Current position: Research Associate, Johns Hopkins University.
9. Herbert Hurwitz, M.D. Non-specific immune responses in colon and pancreatic cancer. 8/93 - 6/96. Current Position: Assistant Professor, Duke University SOM
10. DoYoun Jun, Ph.D. Identification of the T cell targets expressed by renal cell carcinoma. 8/97 - 2/2000. Current Position: Research Associate, Kyungpook National University, Korea.
11. Dung Le, M.D. 6/05-present. Analysis of mesothelin specific antitumor immunity in a mouse model and humans.
12. Dan Laheru, M.D. 6/99 – 6/01. Current Position: Assistant Professor of Oncology, Johns Hopkins University SOM. Received a K23 NIH award with Dr. Jaffee as his mentor.
13. Jean-Pascal Machiels, M.D. Dissection of the mechanism of synergy between tumor vaccines and chemotherapy. 9/98 – 10/00. Current Position: Faculty, Centre anti-cancereux de l'UCL Brussels,Belgium
14. Richard Todd Reilly, Ph.D. Evaluation of antigen based vaccine for the prevention of breast cancer. 9/96 – 2000. Current Position: Assistant Professor in Oncology, Johns Hopkins University SOM.
15. Zhiwei Yu, M.D. Identification of the T cell targets expressed by pancreatic adenocarcinoma. 10/97 – 11/2000. Leave of absence for medical reasons.
16. Xianzheng Zhou, M.D. Identification of the T cell targets expressed by renal cell carcinoma. 4/97 – 8/02. Previously completing a postdoctoral fellowship in Dr. Jaffee's laboratory, recently accepted a position as Assistant Professor in Medicine at the University of Minnesota (8/02).
17. Dung Le, M.D. Evaluation of mechanisms of immune tolerance in HER-2/neu transgenic mice. 7/05-current.
18. Ihid Leao, Ph.D. Evaluation of mesothelin specific T cell responses in a new mouse model of pancreatic cancer. 1/06-present.

Graduate and Medical Students

1. Anne Ercolini. Dissection of the mechanisms of CD8 T cell tolerance in a Her-2/neu transgenic mouse model of breast cancer. 3/97 – 2003. Anne Ercolini completed her Ph.D. work in Immunology in Dr. Jaffee's laboratory in 8/02.
2. Morris Gottlieb, M.D. Evaluation of the mechanisms of CD8 T cell peripheral tolerance. 7/96 – 8/97. Current Position: Medical Resident, Duke Univ. School of Medicine.
3. Alex Huang, M.D., Ph.D. Identification of tumor-specific antigens. 7/92 - 5/97. Current Position: Fellow, Pediatrics, Johns Hopkins University SOM
Johns Hopkins University SOM
4. Diane Weintraub. 6/99 – 2002. Evaluation of the mechanisms of CD4 T cell tolerance.
5. Brian Ladle. 7/01-11/05. Pharmacology graduate student. Tracking of high affinity T cells in tolerized and non-tolerized mice.
6. Robert Georgantas. Thesis Committee Member and Chair, Thesis Defense Committee, 1999-2001.
7. Lukas Pfannestiel, CMM graduate student evaluating CD4+ T cell responses in tolerized HER-2/new mice. September 2002–present.
8. Elizabeth Manning, Pharmacology graduate student working with Dr. Jaffee on evaluating the modulation of the tumor's micro-environment to enhance anti-tumor immunity. September 2003-May 2006.
9. Jennifer Uram. Immunology graduate student working on thesis project. Evaluation of CD8+ HER-2/neu cryptic epitopes in the HER-2/neu transgenic mouse model of mammary tumors. September 2003-present.
10. Eric Lutz. Immunology graduate student working on thesis project. Evaluation of mesothelin specific T cell responses in patients receiving immunization for pancreatic cancer. September 2003-present.
11. Peter Kim. Pharmacology graduate student. Evaluation of the mechanisms of HER-2/neu antibody enhancement of vaccine induced T cell responses in the HER-2/neu transgenic mouse model of mammary tumors. September 2004-present.

Editorial Activities:

Editorial Board Member:

Molecular Cancer Therapeutics – AACR Journal
Cancer Biology and Therapy

2003-2005 Cancer Research-Associate Editor

Journal Reviews:

1992 - present	Cancer Research - Reviewer
1993 - present	Journal of Immunology - Reviewer
1993 - present	Journal of the National Institutes of Health - Reviewer
1993 – present	Journal of Immunotherapy - Reviewer
1997 – present	Human Gene Therapy – Reviewer
1998 – present	Journal of Clinical Oncology - Reviewer
1998 – present	Nature Medicine - Reviewer
1998 - present	Journal of Clinical Investigation – Reviewer
1998 – present	Science- Reviewer
1999 - present	New England Journal of Medicine – Reviewer
2003 – present	The Journal of Gene Medicine – Associate Editor

CLINICAL ACTIVITIES:

Certifications:

1987 - Pennsylvania Bureau of Professional and Occupational Affairs #MD 037496-E

1987 - Drug Enforcement Administration, U.S. Department of Justice

1989 - American Board of Internal Medicine #119426

1989 - Maryland Bureau of Physician Quality Assurance #D38653

1989 - Maryland Division of Drug Control #M29295

1993 - American Board of Internal Medicine/Subspecialty in Oncology

2002 - American Board of Internal Medicine/Subspecialty in Oncology

Service Responsibilities:

Supervisor of Medical Oncology Fellows Clinic

Clinical Trials:

1. Phase I Study of Non-Replicating Autologous Tumor Cell Injections Using Cells Prepared with or without GM-CSF Gene Transduction in Patients with Metastatic Renal Cell Carcinoma. Completed 6/95. Role on project: Principal Investigator of the Laboratory Component of this Study.
2. A Phase I Clinical Trial of Lethally Irradiated Allogeneic Pancreatic Tumor Cells Transfected with the GM-CSF Gene for the Treatment of Pancreatic Adenocarcinoma. Study Completed 6/99. Role on project. Principal Investigator of both the clinical and laboratory components of this study.

3. A Safety and Efficacy Trial of Lethally Irradiated Allegoric Pancreatic Tumor Cells Transfected with the GM-CSF Gene in Combination with Adjuvant Chemoradiation for the Treatment of Adenocarcinoma of the Pancreas. Role on Project. Principal Investigator of both the clinical and laboratory components of this study.
4. A Phase I Vaccine Safety and Chemotherapy Dose-Finding Trial of An Allogeneic GM-CSF-Secreting Breast Cancer Vaccine Given in a Specifically Timed Sequence with Immunomodulatory Doses of Cyclophosphamide and Doxorubicin. Role on Project. Principal Investigator of both the clinical and laboratory components of this study.
5. Phase I Clinical Trial of Vaccine Boosting with Lethally Irradiated Allogeneic Pancreatic Tumor Cells Transfected with the GM-CSF Gene for the Treatment of Pancreatic Cancer. Role on Project. Principal Investigator of both the clinical and laboratory components of this study.
6. Phase II study of GVAX alone versus GVAX plus immune modulating Doses of Cyclophosphamide in Patients with metastatic pancreatic adenocarcinoma. Role on Project. Laboratory PI.
7. Phase II study of an Allogeneic GM-CSF secreting tumor vaccine in sequence with Cyclophosphamide and Erbitux for patients with advanced pancreatic cancer. Role on Project. Laboratory PI.
8. Phase I study of a Monoclonal Antibody to Mesothelin in patients with advanced pancreatic cancer. Collaboration with Morphotek, Inc. Role on Project. Laboratory PI.
9. A Safety and efficacy Trial of Lethally Irradiated Allogeneic Pancreatic Tumor Cells Transfected with GM-CSF Gene in Combination with Erbitux (Cetuximab) for the Treatment of Advanced Pancreatic Adenocarcinoma. Role on Project. Principal Investigator of laboratory component plus this study.

ORGANIZATIONAL ACTIVITIES

Institutional and Departmental Administrative Appointments:

1992-present	Department of Oncology Fellowship Selection Committee.
1993-present	Oncology Center Medical Student Committee.
1994-1995	Oncology Center Faculty Compensation Committee.
1995-1996	Department of Oncology Pharmacology Search Committee.
1996	Oncology Center Faculty Compensation Committee.
1997-1998	Member of the JHOC Executive Board.

1999-present	Member of the JHOC Executive Clinical Research Committee
1999-present	Member of the JHOC Education Committee
2002-2005	Member of the Johns Hopkins School of Medicine Conflict of Interest Committee
2000-2002	Member of the School of Medicine Residency/Fellowship Training Committee
2001-present	The Johns Hopkins School of Medicine Young Investigators' Day Committee
2002-present	Johns Hopkins Cancer Center Faculty Appointments and Promotions Committee
2004-2005	Chair, Clinical Research Committee, Sidney Kimmel Cancer Center at Johns Hopkins
2006-present	Member, Professorial Promotions Committee, Johns Hopkins School of Medicine
2006-present	Deputy Director, Johns Hopkins School of Medicine Clinical and Translational Research Institute

Study Sections:

5/4-6, 1994	NCI Program Grant in Gene Therapy Site Visit Reviewer
1998	NCI Grant Review Committee, RFA CA-94-04, "New Therapeutic Approaches for Breast Cancer".
1995	NCI Experimental Immunology Study Section, Ad hoc
1996	Susan G. Komen Breast Cancer Foundation, Ad hoc 5.
1996 - 2000	NCI Experimental Immunology Study Section, Member
1999 – 2000	NIH RAID Program Special Review Group
1998 - present	Ad Hoc Reviewer for The Dutch Cancer Society.
2001-2004	NCI Parent Subcommittee D – Translational Research Program Grant Review Group
1/22-1/24/2003	NCI Sponsored Tumor Immunology Workshop Think Tank

2005-2010 NCI Board of Scientific Counselors

External Advisor:

2001- 2003	Program Project Grant: “Cytokine Gene Therapy for Cancer”, University of Pittsburgh Medical Center
2000-2001	NCI Special Emphasis Panel Member, Program Review Group for Pancreatic Cancer
5/2001	Consultant, Fred Hutchinson Cancer Center, Review of Translational Research Program in Solid Tumors
9/2003 - present	Roswell Park Cancer Institute’s External Advisory Board, Buffalo, NY
7/2004-present	Fox Chase Cancer Center’s Ovarian Cancer SPORE External Advisory Board, Philadelphia, PA

Memberships in Professional Societies:

American Association for Cancer Research
American Association for the Advancement of Science
American Society of Clinical Oncology
American Association of Immunologists
Society of Biological Therapy

National Committees:

American Association for Cancer Research:

1999- present	Annual Meeting: Educational Sessions and Methods Workshops Planning Committee.
1999-2000	Annual Meeting: Clinical Trials Workshop Educational Session Co- organizer.
1999-2001	Annual AACR Meeting Meet the Expert in Cancer Vaccines
2001-present	Women in Cancer Research Charlotte Friend Memorial Committee
2003-2004	AACR Program Committee’s Subcommittee Chairperson
2003-2006	Women In Cancer Research Council (WICR) AACR
2005-2008	Scientific Program Committee, ASCO
2006	Chairperson of the Clinical Immunology/Biological Therapy Subcommittee of the Clinical Research Section of the 2006 AACR Program Committee

NIH:

1998	Search Committee Chief, Laboratory of Immunotherapy. National Institute on Aging.
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RECOGNITION

Honors

1977 - 1981	Brandeis University - Dean's List
1981	Brandeis University - Magna cum laude with highest honors in Biology/Immunology
1989	American Cancer Society Clinical Fellow
1992	Stetler Award
1992	American Cancer Society Research Fellowship Award
1992	American Society of Clinical Oncology Young Investigator Award
1992	Physician-Scientist Award, NIH
1992	Clinician-Scientist Award, Johns Hopkins School of Medicine
	National Kidney Cancer Career Development Award
1998	JHU Department of Oncology - Director's Award for Outstanding Teaching
2001	JHU Department of Oncology - Director's Award for Outstanding Teaching
2002	Recipient of the Dana and Albert Broccoli Endowed Chair in Oncology

Invited Lectures and Symposia

University Seminars

1. University of Southern California, Institute for Genetic Medicine and the Kenneth Norris Jr. Comprehensive Cancer Center Symposium on Gene Therapy for Human Disease: Basic Science and Clinical Applications. Friday, November 6, 1992.
2. Johns Hopkins Oncology Center. Biology of Cancer Course. New Approaches to Cancer Therapy. Baltimore, Maryland. May 9, 1994.
3. Department of M.C.D. Biology, University of Colorado, and Nexagen, Inc. Treating Cancer through Immune Recruitment. Vaccine Therapy of Cancer: Practice and Promise. Boulder, Colorado. November 12-13, 1994.
4. University of Cincinnati, Molecular Medicine and the Treatment of Cancer. Cincinnati, Ohio, November 10-11, 1995.
5. Johns Hopkins Oncology Center. Diagnosis and Treatment of Neoplastic Disorders. Vaccine strategies for the treatment of adenocarcinoma of the pancreas. Baltimore, Maryland. April 3-4, 1997.
6. Roswell Park Cancer Institute, Staff Conference. Buffalo, New York. April 9, 1997.
7. University of Maryland Hematology and Oncology Grand Rounds. November 1997.
8. Johns Hopkins Department of Allergy and Immunology Grand Rounds. January 9, 1998.
9. Johns Hopkins Department of Pathology Grand Rounds. February 19, 1998

10. Johns Hopkins Department of Cell Biology Ground Rounds. March, 1998.
11. The Rockefeller University. Anti-Cancer Proteins and Drugs: Structure, Function and Design. "Immunology of Cancer" November 6-9, 1998.
12. University of Nebraska Oncology Grand Rounds. May 12, 1999.
13. Roswell Park Cancer Institute Grand Rounds. July 23, 1999.
14. Johns Hopkins Oncology Center. Diagnosis and Treatment of Neoplastic Disorders. Development of vaccines for the treatment for pancreatic cancer: Bench to bedside. Baltimore, Maryland. April 7, 2000.
15. University of Pennsylvania Grand Rounds. Engineering immune responses to eradicate cancer: From mice to man. Philadelphia, PA, May 17, 2000.
16. University of Connecticut Health Center, Center for Immunotherapy Seminar Series. Farmington, CT. October 19, 2000.
17. University of Michigan Medical Center Grand Rounds. Ann Arbor, Michigan April 27, 2001.
18. University of Alabama Birmingham. Birmingham, Alabama. Vaccines for Cancer Treatment: From Mice to Men. May 2, 2001
19. Fred Hutchinson Cancer Research Center, Seattle, Washington, Vaccines for Cancer Treatment: From Mice to Men. May 7, 2001
20. Scientific Sessions of the 183rd meeting of the Interurban Clinical Club. Vaccines for Cancer Treatment: From Mice to Men. 2001.
21. Johns Hopkins Grand Rounds Department of Medicine. Pancreatic Cancer: Practice and Promise. October 12, 2001
22. Vanderbilt-Ingram Cancer Center, Speaker at the Experimental Therapeutics Program Seminar, February 4, 2002.
23. Congressional Briefing "New Approaches to Cancer Treatment and Prevention: Genomics and Proteomics", Capitol Hill, Washington, D.C., February 12, 2002.
24. H. Lee. Moffitt Cancer Center and Research Institute, Speaker at Ground Rounds "Vaccines for Cancer Treatment: From Mice to Men", March 8, 2002.
25. NIH Seminar Presentation April 18, 2002.
26. Gordon Research Conference on Mammary Gland Biology 2002 in Luca, Italy, Speaker: "Combinatorial Therapies for Breast Cancer Treatment", April 28, 2002.

27. Annual NCI sponsored SPORE Meeting – Organizer of the Break Out Session on Immunotherapy, and Plenary Session Speaker: “Vaccines for Pancreatic Cancer: An Update”, July 14-16, 2002.
28. Invitation to speak at the Institute of Medicine, Special Emphasis Panel on Translation Research. Topic: Barriers to Translation Research. July 17, 2002.
29. Eighth Annual Symposium of the Penn State Cancer Institute, Speaker: “Vaccines for Cancer Treatment and Prevention: From Mice to Men”. October 31, 2002
30. Voyage and Discovery Lecture Series, Speaker-Johns Hopkins University, Homewood Campus. March 25, 2003.
31. Invitation to speak at the 34th International Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo, Japan. Topic: From Genes to Vaccines for Pancreatic Cancer. November 11-13, 2003.
32. Invitation to speak at the University of Nebraska. Topic: “Vaccines for Cancer Treatment: From Mice to Men”. Omaha, NE. February 25, 2004.
33. Invited to give a lecture at Howard University. Topic: “Cancer Immunology”. Jointly sponsored by Johns Hopkins University and Howard University, Washington, D.C., March 24, 2004.
34. Invitation to speak at the University of North Carolina/Chapel Hill. Topic: “Module Immune Tolerance on mice and men” February 2005
35. Invitation to speak at the Vanderbilt University’s Experimental Therapy Lecture Series, May 2005. Topic: Genes to vaccines for pancreatic cancer: How the molecular biology revolution has influenced cancer immunology.
36. Invitation to speak at the University of Pennsylvania’s Molecular Studies in Digestive and Liver Disease Seminar, November 2006 Topic: Genes, Vaccines, and Immune Checkpoints: Raising Hope for Pancreatic Cancer Therapy.
37. Invitation to speak at the University of South Florida College of Medicine and H. Lee Moffitt Cancer Center and Research Institute. Topic: “Tipping the Immune System Balance in Favor of Effective Anti-Cancer Therapy” January 25, 2007
38. Invitation to speak at the The Society of Surgical Oncology, Inc, Washington, D.C. Topic: “Adjuvant Therapy for Pancreatic Cancer” March 17, 2007

National and International Meetings

1. Keystone Symposium on Cellular Immunity and Immunotherapy of Cancer, Workshop: Role of MHC Molecules in Tumor Recognition and Rejection. Toas, New Mexico. March 19, 1993.
2. First International Conference on Engineered Cancer Vaccines and AIDS Vaccines. Analysis of the Immune Response Induced by Tumors Engineered to Secrete Cytokines. San Francisco, CA. September 30 -October 2, 1993.
3. American Association for Cancer Research Conference on Molecular Approaches to Cancer Immunotherapy. Identification of Immunodominant Tumor Specific Antigens and Their Use in Antigen Specific Immunotherapy. Ashville, North Carolina. November 9, 1993.
4. U.S./Japan workshop on "Cell Biology of the Host Anti-Tumor Immune Response". Identification of Immunodominant Tumor-Specific Antigens and their Use in Antigen-Specific Immunotherapy. Bethesda, Maryland. January 10-12, 1994.
5. International Conference on Gene Therapy and Vaccines for Cancer. Analysis of the Immune Responses Induced by Cytokine-Secreting Tumor Vaccines. Washington, D.C. January 26-27, 1994.
6. The Third International Symposium on the Biology of Renal Cell Carcinoma at the Cleveland Clinics. Analysis of the Immune Responses Induced by Cytokine-Secreting Tumor Vaccines: Extrapolation to Clinical Trials. Cleveland, Ohio. March 7-8, 1994.
7. Bio/Technology. Clinical Trials: New Paradigms for Success. Gene Therapy in Practice: A View from the Clinic. Washington, D.C. June 20-21, 1995.
8. American Society of Nephrology. 1994 Basic Science Symposia. Cytokine Gene Therapy of Renal Cancer. Orlando, Florida. October 26-30, 1994.
9. Association of Graduates of the Faculty of Medicine at the Federico Villarreal National University International Meeting on Cancer Treatment. New Approaches for Cancer Therapy. Lima, Peru. November 16-19, 1994.
10. Immune Monitoring of Cancer Vaccine Clinical Trials. NIH Campus. Bethesda, MD. April 12, 1995.
11. Cancer Vaccines. Cambridge Healthtech Institute. Arlington, Virginia. June 5-6, 1995.
12. Genomic Science Series Conference on Gene Therapy. Hilton Head, SC, May 9-12, 1996.
13. American Association of Immunologists. Chairperson - Minisymposium on Antigen Presentation and Tumor Immunity. New Orleans, Louisiana. June 2-6, 1996.

14. Berzelius Symposium XXXIV on Virus as Target for Cancer Prevention and Therapy. June 12-14, 1996.
15. National Kidney Cancer Association National Meeting. Chicago, Illinois. July 20, 1996.
16. American College of Surgeons Annual Spring Meeting. April 26-29, 1998.
17. Ask the Experts: AACR Annual Meeting, Progress and New Hope in the Fight Against Cancer. San Francisco, CA, April 1, 2000.
18. The Lustgarten Foundation for Pancreatic Cancer Research: Workshop on Novel Approaches for the Treatment of Pancreatic Cancer. April 26, 1999.
19. National Breast Cancer Coalition Think Tank. Apsen, Colorado. June 30 – July 4, 1999.
20. NCI Sponsored Meeting on Tumor Escape From Immune Recognition (Co-Organizer). 8/22 – 23/99.
21. NCI Sponsored Pancreatic Cancer Think Tank, Park City, Utah. Speaker and Organizer of Workshops on Therapy. September 16 – 19, 1999.
22. AACT, NCI and EORTC sponsored Molecular Targets in Cancer Therapeutics: Discovery, Development, and Clinical Validation. November 16-19, 1999
23. Second Annual Colloquium on Cancer Vaccines and Immunotherapy. Walkers Cay, Bahamas. March 8-11, 2000.
24. Eighth Annual Advocacy Training Conference for the National Breast Cancer Coalition Fund. Washington, D.C., April 29, 2000.
25. American Society of Clinical Oncology. Speaker at Educational Session. Update on the Treatment of Pancreas Cancer. New Orleans, LA. May 20-23, 2000.
26. 12th Annual Cancer Progress Conference. New York, New York. October 3, 2000.
27. Visiting Professor at the Ludwig Institute in Belgium. June 17, 2001
28. Lustgarten Foundation for Pancreatic Cancer Research Third Annual Scientific Conference at JHMI – Co-Chair. June, 2001.
29. Co-chair of the NIH Sponsored SPORE Meeting Breakout Session on Cancer Vaccines. Westfield, Virginia, July 10-11, 2001.

30. Grand Rounds. Vaccines for Pancreatic Cancer: From Mice to Men. Mayo Clinic, Rochester, MN, July 26, 2001.
31. Pancreas Cancer 2001, "Systemic Immunotherapy", Seattle, Washington, August 24-25, 2001.
32. Williamsburg Bioprocessing Foundation, "The Design and Operation of a GMP Facility at Johns Hopkins University", Rockville, MD, October 16, 2001.
33. Society for Biological Therapy in cooperation with the CTEP Vaccine Working Group-NIH and the United States FDA international workshop, NIH Campus, Bethesda, MD, November 8, 2001.
34. The 16th Annual Scientific Meeting for the Society for Biological Training, Natcher Auditorium, Bethesda, MD, November 9th –11th 2001.
35. Vanderbilt-Ingram Cancer Center, Speaker at the Experimental Therapeutics Program Seminar, February 4, 2002.
36. Congressional Briefing, "New Approaches to Cancer Treatment and Prevention: Genomics and Proteomics", Capitol Hill, Washington DC. February 12, 2002.
37. H. Lee Moffitt Cancer Center & Research Institute, Speaker for Grand Rounds "Vaccines for Cancer Treatment: From Mice to Men", March 8, 2002.
38. AACR 2002, 93rd Annual Meeting, Co-Chairperson, "Advances in Tumor Specific Immunity Minisymposium Immunology/Experimental & Preclinical 3." April 6-10, 2002.
39. NIH, "Seminar Presentation", April 18, 2002.
40. Gordon Research Conferences, Mammary Gland Biology Conference 2002, Speaker, Session VI, "Combinatorial therapies for breast cancer treatment". April 28, 2002.
41. NCI Sponsored Tumor Immunology Think Tank, Bethesda, Maryland. January 22 – 24, 2003.
42. The Seventh US-Japan Cellular and Gene Therapy Conference. Bethesda, Maryland. March 4, 2004.
43. 95th AACR Annual Meeting. Roundtable discussion leader during the WICR Council sponsored Leila Diamond Memorial Networking Breakfast. Orlando, FL. March 29, 2004.
44. 95th AACR Annual Meeting. Served as a mentor during a special program for High School Students entitled, "The War Against Cancer: The Task for the Next Generation". Orlando, FL. March, 2004.

45. 95th AACR Annual Meeting. Roundtable mentor during the Associate Member Grant Writing Workshop. Orlando, FL. March, 2004.
46. 95th AACR Annual Meeting. Roundtable discussion leader during the Minorities in Cancer Research Council sponsored Professional Advancement Roundtable entitled, "Navigating the Road to a Successful Career in Cancer Research". Orlando, FL. March, 2004.
47. Lustgarten Foundation for Pancreatic Cancer Research, Advances and Challenges Conference. Topic: "Pancreatic Cancer Vaccines: From Mice to Men". San Francisco, CA. June, 2004.
48. 96th AACR annual meeting. Conference Topic: "Cancer Vaccines Complementing Anticancer Therapy", Anaheim, California, April 2005.
49. Co-chair, International Society for Biological Therapy of Cancer annual meeting, Symposium: "Overcoming Immunosuppression / Tolerance in Cancer Patients", Alexandria, VA, November, 2005.
50. International Society for Biological Therapy of Cancer annual meeting, Symposium: "Recruitment of high avidity T Cells to the Anti-tumor immune response.", Alexandria, VA, November, 2005
51. Cancer Vaccine Consortium: "Multi-Targeted immune Based Therapies: Science Driving Clinical Practice", Washington, DC, November, 2006
52. American Association for Cancer Research: "Genes, Vaccines, and Immune Response: An All Out Attack on Pancreatic Cancer.", Miami, FL, November, 2006.

OTHER PROFESSIONAL ACCOMPLISHMENTS

1. Established and Direct the Johns Hopkins Oncology Center Cell Processing and Gene Therapy cGMP Facility. Purpose of facility: To conduct clinical trials employing cellular and genetic based vaccine therapies for the treatment of cancer.

NEW INVENTIONS.

1. Identification of Mesothelin as a new tumor antigen expressed by pancreatic adenocarcinomas (submitted 6/02).

EXHIBIT 2

Mesothelin-specific CD8⁺ T Cell Responses Provide Evidence of In Vivo Cross-Priming by Antigen-Presenting Cells in Vaccinated Pancreatic Cancer Patients

Amy Morck Thomas,¹ Lynn M. Santarsiero,¹ Eric R. Lutz,^{1,2} Todd D. Armstrong,¹ Yi-Cheng Chen,¹ Lan-Qing Huang,¹ Daniel A. Laheru,¹ Michael Goggins,³ Ralph H. Hruban,³ and Elizabeth M. Jaffee^{1,2,3}

¹Department of Oncology, ²The Graduate Program in Immunology, and ³Department of Pathology, The Sidney Kimmel Cancer Center, Johns Hopkins University, Baltimore, MD 21231

Abstract

Tumor-specific CD8⁺ T cells can potentially be activated by two distinct mechanisms of major histocompatibility complex class I-restricted antigen presentation as follows: direct presentation by tumor cells themselves or indirect presentation by professional antigen-presenting cells (APCs). However, controversy still exists as to whether indirect presentation (the cross-priming mechanism) can contribute to effective in vivo priming of tumor-specific CD8⁺ T cells that are capable of eradicating cancer in patients. A clinical trial of vaccination with granulocyte macrophage-colony stimulating factor-transduced pancreatic cancer lines was designed to test whether cross-presentation by locally recruited APCs can activate pancreatic tumor-specific CD8⁺ T cells. Previously, we reported postvaccination delayed-type hypersensitivity (DTH) responses to autologous tumor in 3 out of 14 treated patients. Mesothelin is an antigen demonstrated previously by gene expression profiling to be up-regulated in most pancreatic cancers. We report here the consistent induction of CD8⁺ T cell responses to multiple HLA-A2, A3, and A24-restricted mesothelin epitopes exclusively in the three patients with vaccine-induced DTH responses. Importantly, neither of the vaccinating pancreatic cancer cell lines expressed HLA-A2, A3, or A24. These results provide the first direct evidence that CD8 T cell responses can be generated via cross-presentation by an immunotherapy approach designed to recruit APCs to the vaccination site.

Key words: immunotherapy • antigen • cancer vaccine • epitopes • T lymphocytes

Introduction

A major goal of vaccine development is to design immunization strategies that activate CD8⁺ T cells. CD8⁺ T cells are the effector cells most capable of directly recognizing and lysing their target, whether it be a virally infected cell or a tumor cell. Activation of CD8⁺ T cells requires target cell presentation of antigen on MHC class I (1). In a host with cancer, tumor cells can present endogenous MHC class I-restricted antigens to CD8⁺ T cells by direct presentation. However, the APCs of the host, rather than the tumor cells themselves, can also process and present acquired tumor antigens captured from the tumor's microenvironment, to prime CD8⁺ T cells. The mechanism of transferring exog-

enously acquired antigens from the APC endocytic processing and presentation pathway into the cytosol for processing and presentation via the proteasome (the endogenous processing and presentation pathway) is referred to as cross-priming (2–10).

Many preclinical studies have demonstrated that APCs have the ability to capture dying cells, and process and present captured antigens expressed by these cells to CD8⁺ T cells (5, 8, 11–16). Several groups have shown that the DC, in particular, exhibits efficient cross-priming in both human and mouse models in vitro (6, 17). Although cross-priming has been confirmed as a mechanism by which CD8⁺ T cells can be primed in vitro, controversy still exists concerning the efficiency of this mechanism at priming

Address correspondence to Elizabeth Jaffee, Dept. of Oncology, The Sidney Kimmel Cancer Center at Johns Hopkins, The Bunting-Blaustein Cancer Research Bldg., Rm. 4M07, 1650 Orleans St., Baltimore, MD 21231. Phone: (410) 955-2957; Fax: (410) 614-8216; email: ejaffee@jhmi.edu

Abbreviations used in this paper: DTH, delayed-type hypersensitivity; SAGE, serial analysis of gene expression.

CD8⁺ T cells *in vivo* (18). In several murine tumor models, Zinkernagel et al. found that, whereas MHC class II-restricted antigens were efficiently cross-presented, CTL activation occurred exclusively via direct presentation of MHC class I-restricted antigens by the tumor (18). A better understanding of the role of the cross-priming mechanism in the induction of CD8⁺ T cells *in vivo* will have important implications for future vaccine development.

Several cancer vaccine approaches under clinical development specifically aim to recruit and activate DCs as a first step in priming both CD4⁺ and CD8⁺ T cells (19, 20). The unique capacity of DCs to stimulate tumor-reactive T cell lines from cancer patients emphasizes the importance of the cell type in recruiting cryptic populations of tolerant or low affinity T cells into an antitumor response (21). In particular, whole cell vaccine approaches have already demonstrated that the APCs of the host, rather than the vaccinating tumor cells themselves, can prime both CD4⁺ and CD8⁺ T cells that are capable of generating systemic antitumor immunity against transplanted murine tumors *in vivo* (14, 22, 23). It has been more difficult to prove that cross-priming is involved in the induction of clinically meaningful CD8⁺ T cell responses in patients. The major impediment has been the lack of correlation of immunization and the induction of T cell responses in reported studies.

In a recently completed phase I trial, a vaccine consisting of two allogeneic, GM-CSF-secreting pancreatic tumor cell lines induced a dose-dependent delayed-type hypersensitivity (DTH) response to autologous tumor cells in 3 out of 14 patients (24). A whole cell tumor vaccine approach allows for polyvalent immunizations under circumstances where relevant tumor rejection antigens have not yet been identified. This allogeneic GM-CSF-secreting pancreatic tumor vaccine was specifically designed to test whether GM-CSF can recruit APCs, in particular DCs, to the site of vaccination and subsequently prime CD8⁺ T cells by the cross-priming mechanism. To determine whether this vaccine induced CD8⁺ T cell responses and to study the mechanism of activation of these responses, we have developed a functional genomic approach that uses immunized lymphocytes from vaccinated patients to identify immunologically recognized tumor-associated antigens from among genes overexpressed in the relevant tumor type. Here, we identify a pancreatic tumor-associated antigen, mesothelin, as a relevant target of vaccine-induced CD8⁺ T cell responses. We use these responses to directly evaluate the capacity of the GM-CSF-transduced vaccines to induce cross-priming in pancreatic cancer patients.

Materials and Methods

Identification of Candidate Genes and Epitope Selection. Serial analysis of gene expression (SAGE) was used to identify mesothelin as one of the genes overexpressed in pancreatic cancer cell lines and fresh tissue (25, 26). Two computer algorithms "BLMAS" (27) and "SYFPEITHI" (28) that are available to the general public and accessible through the internet were used to predict peptides that bind to HLA-A2, A3, and A24 molecules.

Peptides and T2 Cell Lines. All peptides were purified to >95% purity and synthesized by Macromolecular Resources according to the following published sequences: M1 peptide₍₅₈₋₆₆₎ GILGFVFTL, derived from influenza matrix protein (29); mesothelin A2₍₂₀₋₂₈₎ peptide SLLFLFLSL; mesothelin A2₍₅₃₀₋₅₃₈₎ peptide VLPLTVAEV, identified using the available databases; and HIV-gag A2 peptide SLNTVATL₍₇₅₋₈₃₎ (30), which contains an HLA-A2 binding motif. Mesothelin A3₍₈₃₋₉₁₎ peptide ELAVLAQK, mesothelin A3₍₂₂₅₋₂₃₃₎ peptide ALQGGGPPY, and HIV-NEF A3₍₉₄₋₁₀₃₎ peptide QVPLRPMTYK (31) contain an HLA-A3 binding motif. Mesothelin A24₍₄₃₅₋₄₄₃₎ peptide FYPGYLCSL, mesothelin A24₍₄₇₅₋₄₈₃₎ peptide LYPKARLAF, and tyrosinase peptide AFLPWHRLE₍₂₀₆₋₂₁₄₎ (32) contain an HLA-A24 binding motif. The mesothelin A1₍₃₀₉₋₃₁₇₎ peptide EIDESLIFY was used as a negative control peptide and contains an HLA-A1 binding motif. Stock solutions (10 mg/ml) of peptides were prepared in 100% DMSO (JT Baker) and further diluted in cell culture medium to yield a final peptide concentration of 10 ng/ml for each assay. The T2 cells are a human B and T lymphoblast hybrid that only expresses the HLA-A*0201 allele (33). T2 cells are TAP deficient and, therefore, fail to transport newly processed HLA class I binding epitopes from the cytosol into the endoplasmic reticulum, where these epitopes would normally bind to nascent HLA molecules and stabilize them for expression on the cell surface (33). The T2-A3 are T2 cells genetically modified to express the HLA-A*0301 allele and were a gift from W. Storkus (University of Pittsburgh, Pittsburgh, PA; reference 34). The T2-A24 are T2 cells genetically modified to express the HLA-A24 allele. The HLA-A24 gene was a gift from P. Robbins (National Cancer Institute, Bethesda, MD; reference 32). T2 cells were grown in suspension culture in RPMI 1640 (GIBCO BRL), 10% FBS (Hyclone) supplemented with 200 μ M L-glutamine (JRH Biosciences), 50 U/ μ g/ml of Pen/Strep (Sigma-Aldrich), 1% NEAA (Sigma-Aldrich), and 1% Na-Pyruvate (Sigma-Aldrich) in 5% CO₂ at 37°C.

Peptide/MHC Binding Assays. T2 cells expressing the HLA molecule of interest were resuspended in AimV serum-free media (GIBCO BRL) to a concentration of 10⁶ cells/ml and pulsed with β -2 microglobulin (β_2 -M) plus peptide at concentrations ranging from 0 to 225 μ g/ml of peptide at room temperature overnight. The level of stabilized MHC on the cell surface of the T2 and T2-A24 cells was analyzed by direct staining of cell samples with unlabeled anti-class I mAb W6/32 and a goat anti-mouse FITC-labeled IgG2a secondary antibody. The level of stabilized MHC on the cell surface of the T2-A3 cells was analyzed by direct staining of cell samples with unlabeled anti-HLA-A3 mAb GAPA3 and a goat anti-mouse FITC-labeled IgG2a secondary antibody. Viable cells, as determined by exclusion of propidium iodide, were analyzed by flow cytometry on a dual laser FACSCalibur™ (Becton Dickinson) using FlowJo analysis software (Treestar). Data are expressed as an increase in mean fluorescence intensity (Δ MFI) of cells with each peptide compared with that determined for cells without peptide or a negative control peptide.

PBLs and Donors. Peripheral blood (100 cc prevaccination and 28 d after each vaccination) were obtained from all 14 patients who received an allogeneic GM-CSF-secreting pancreatic tumor vaccine as part of a previously reported phase I vaccine analysis (24). Informed consent for banking lymphocytes to be used for this antigen identification study was obtained at the time of patient enrollment into the study. Pre- and postvaccine PBLs were isolated by density gradient centrifugation using Ficoll-Hypaque (Amersham Biosciences). Cells were washed twice with serum-free RPMI 1640. PBLs were stored frozen at -140°C in 90% AIM-V media containing 10% DMSO.

Enrichment of PBLs for CD8⁺ T Cells. CD8⁺ T cells were isolated from thawed PBLs using magnetic cell sorting of human leukocytes as per the manufacturer's directions (MACS; Miltenyi Biotec). Cells were fluorescently stained with CD8-PE antibody (Becton Dickinson) to confirm that the positive population contained CD8⁺ T cells and analyzed by flow cytometry. This procedure consistently yielded >95% CD8⁺ T cell purity.

CD8⁺ M1-specific T Cell Lines. M1-specific T cell lines were generated by repeated *in vitro* stimulation of HLA-A*0201⁺ PBLs initially with irradiated autologous dendritic cells followed by irradiated autologous EBV-transformed B cells, both pulsed with the HLA-A*0201-restricted epitope. T cells were stimulated at a 1:2 T cell/EBV cell ratio in T cell media consisting of RPMI 1640, 10% human serum (pooled serum collected at the Johns Hopkins University Hemapheresis Unit), 200 μ M L-Glutamine, 50 U/ μ g/ml Pen/Strep, 10 mM Hepes (GIBCO BRL) supplemented with 60 international units IL-2/ml (R&D Systems), and 10 ng/well IL-7 (R&D Systems). This line was used as a positive control T cell line in all assays.

ELISPOT Assay. Multiscreen 96-well filtration plates (Millipore) were coated overnight at 4°C with 60 μ l/well of 10 μ g/ml anti- γ mouse Mab 1-D1K (Mabtech). Wells were washed three times each with PBS and blocked for 2 h with T cell media. 10⁵ T2 cells pulsed with 10 ng/ml of peptide in 100 μ l of T cell media were incubated overnight with 10⁵ thawed PBLs that were purified to select CD8⁺ T cells in 100 μ l T cell media on the ELISPOT plates in replicates of six. The plates were incubated overnight at 37°C in 5% CO₂. Cells were removed from the ELISPOT plates by washing six times with PBS + 0.05% Tween 20 (Sigma-Aldrich). Wells were incubated for 2 h at 37°C in 5% CO₂ using 60 μ l/well of 2 μ g/ml of biotinylated Mab anti- γ 7-B6-1 (Mabtech). The avidin peroxidase complex (Vectastain ELITE ABC kit; Vector Laboratories) was added after washing six times with PBS/Tween 0.05% at 100 μ l/well and incubated for 1 h at room temperature. AEC substrate solution (3-amino-9-ethylcarbazole) was added at 100 μ l/well and incubated for 4–12 min at room temperature. Color development was stopped by washing with tap water. Plates were dried overnight at room temperature, and colored spots were counted using an automated image system ELISPOT reader (Axioplan2; Carl Zeiss Microimaging, Inc.).

Flow Cytometry. The cell lines were washed twice and resuspended in FACS[®] buffer (HBSS supplemented with 1% PBS, 2% FBS, and 0.2% sodium azide), stained with mouse monoclonal mesothelin (CAK1; Signet Laboratories) followed by FITC-labeled goat anti-mouse IgG1 (BD Biosciences) for flow analysis in a FACScan[™] analyzer (BD Immunocytometry Systems).

***In Vitro* Generation of Tumor-reactive CTLs.** Purified monocytes were cultured for 4 d in the presence of 100 ng/ml recombinant human GM-CSF (R&D Systems) and 10 ng/ml rhIL-4 (R&D Systems) in complete RPMI 1640 medium (35). The monocytes were activated overnight by incubation with 0.5 μ g/ml LPS (Sigma-Aldrich). The tissue culture-generated monocytes were pulsed with 30 μ g/ml of synthetic peptides together with 3 μ g/ml β_2 -M (Sigma-Aldrich) in PBS containing 1% human serum albumin (Sigma-Aldrich) for 4 h at room temperature. The peptide-pulsed DCs were washed twice, irradiated (4,200 rad), and mixed with autologous CD8⁺ T cells (purified with antibody-coated magnetic beads by positive selection; Miltenyi Biotec) at a 1:10 (DC/T cell) ratio. This medium was supplemented with 10 ng/ml rhIL-7 (R&D Systems). 1 d later, 60 international units/ml rhIL-2 (R&D Systems) were added to the cultures to increase the efficiency of CTL induction. Approx-

imately every 10 d, the T cell cultures were restimulated with irradiated peptide-pulsed autologous DCs as previously mentioned, adding rhIL-7 and rhIL-2 on the same day. The cytotoxicity assays were performed after three rounds of peptide stimulation.

Chromium Release Assay. 10⁶ target cells were labeled in 100 μ l complete medium and 100 μ Ci ⁵¹Cr (Amersham Biosciences) at 37°C for 1–1.5 h (36). To determine the mesothelin-specific lysis from patient 13 CD8⁺ T cell line, ⁵¹Cr-labeled target cells (3 \times 10³) were added to varying concentrations of the CD8⁺ T cell line in a total of 200 μ l in a v-bottom 96-well plate for 4 h at 37°C. Each data point was performed in triplicate and averaged. Data are expressed as percentage of specific lysis = (measured release – spontaneous release) / (maximum release – spontaneous release) \times 100. The spontaneous release ranged between 10 and 15% of the total label incorporated into the cells. For HLA-blocking studies, either the pan-HLA antibody W6/32 (HB-95; American Type Culture Collection) or the isotype matched anti-*Schistosoma mansoni* antibody, MBL (HB-193; American Type Culture Collection) were added to the target cells (50 μ g/ml) for 30 min at 37°C before adding the T cells.

Results

Vaccination with an Allogeneic Pancreatic Tumor Vaccine Induces Mesothelin-specific CD8⁺ T Cells. To determine whether the cross-priming mechanism is functional and efficient in inducing CD8⁺ T cells in pancreatic cancer patients receiving an allogeneic, GM-CSF-secreting vaccine, we first needed to identify pancreatic tumor antigens against which vaccine-induced immune responses are elicited. A growing number of genes shown to be differentially expressed in pancreatic adenocarcinomas using SAGE have been tabulated and reported (25, 26, 37). We screened this SAGE analysis database to identify genes that can also serve as potential immune targets for the majority of pancreatic adenocarcinoma patients. We focused specifically only on those genes that were nonmutated, overexpressed by the majority of pancreatic cancer patients, and overexpressed by the vaccine cell lines (all being important requirements for evaluating CD8⁺ T cell cross-priming). One gene that met all three criteria was mesothelin (25), the focus of this paper. We used the combination of two public-use computer algorithms (27, 28, 35) to predict peptide nonamers that bind to three common HLA class I molecules. Both computerized algorithms score candidate epitopes based on amino acid sequences within a given protein that have similar binding motifs to previously published HLA-binding epitopes. We synthesized the top two ranking mesothelin epitopes for HLA-A2, HLA-A3, and HLA-A24 favored by both algorithms because at least one of these three HLA class I molecules is expressed by each of the 14 patients that were treated in the vaccine paper (24). The human T2 cell line, which expresses empty MHC class I molecules on its surface because it is TAP transporter deficient, was used to confirm epitope binding to MHC class I (33). Binding of these epitopes to their respective HLA class I molecule was confirmed by pulsing TAP-deficient T2 cells that expressed the corresponding HLA class I molecule (T2-A2, T2-A3, or T2-A24 cells). As shown in Fig. 1 A, pulsing of two me-

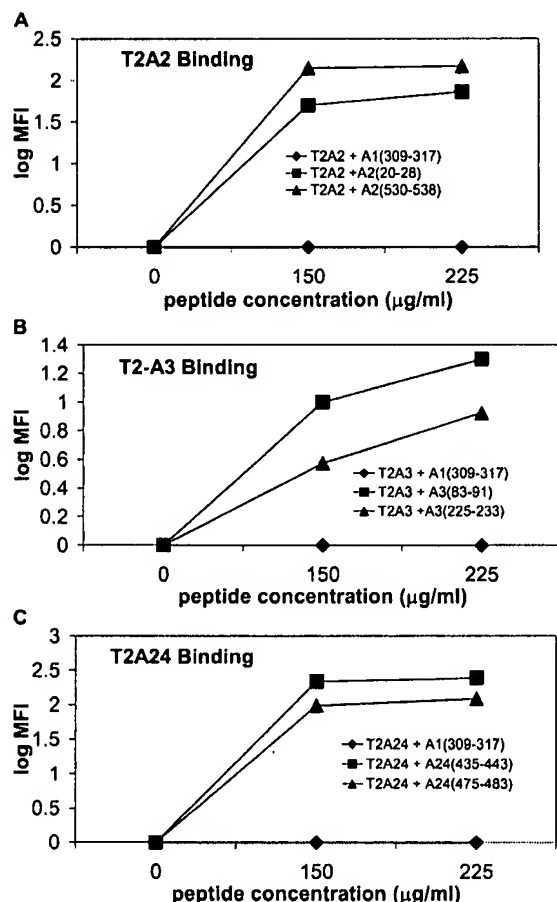


Figure 1. T2 binding assay identifies mesothelin protein-derived epitopes that bind to HLA-A2, A3, and A24 molecules. T2 cells were pulsed with 0–225 µg/ml of peptide overnight at room temperature before analysis by flow cytometry. (A) T2 cells expressing HLA-A2 and pulsed with either a mesothelin A1_(309–317) peptide (closed diamond), mesothelin A2_(20–28) (closed square), and mesothelin A2_(530–538) (closed triangle). (B) T2 cells genetically modified to express HLA-A3 and pulsed with either mesothelin A1_(309–317) peptide (closed diamond), mesothelin A3_(83–91) (closed square), and mesothelin A3_(225–233) (closed triangle). (C) T2 cells genetically modified to express HLA-A24 and pulsed with either mesothelin A1_(309–317) peptide (closed diamond), mesothelin A24_(435–443) (closed square), and mesothelin A24_(475–483) (closed triangle).

sothelin-derived epitopes predicted to bind to HLA-A2 allowed for detection of HLA-A2 on the cell surface of T2-A2 cells by flow cytometry after staining with the HLA class I-specific antibody, W6/32. In contrast, T2 cells pulsed with a mesothelin epitope predicted to bind to HLA-A1 do not stain with the same antibody. Binding of T2 cells pulsed with two candidate mesothelin-derived HLA-A3 and two candidate HLA-A24 epitopes demonstrated similar results (Fig. 1, B and C, respectively).

To determine if mesothelin is recognized by CD8⁺ T cells, we screened antigen-pulsed T2 cells in a quantitative ELISPOT-based assay using pre- and postvaccination CD8⁺ T cell-enriched PBLs from the 14 patients treated previ-

ously with the allogeneic, GM-CSF-secreting pancreatic tumor vaccine. Previously, we reported the association of in vivo postvaccination DTH responses to autologous tumor in three out of eight patients receiving the highest two doses of vaccine. PBLs obtained before vaccination and 28 d after the first vaccination were initially analyzed. T2-A3 cells pulsed with the two A3 binding epitopes were incubated overnight with CD8⁺ T cell-enriched lymphocytes isolated from the peripheral blood of patients 11 (an A3 non-DTH responder) and 13 (an A3 DTH responder), and analyzed using an IFN-γ ELISPOT assay. The ELISPOT assay was chosen because it requires relatively few lymphocytes, is among the most sensitive in vitro assays for quantitating antigen-specific T cells, and correlates the number of antigen-specific T cells with function (cytokine expression; references 38–40). Induction of mesothelin-specific T cells was detected 28 d after vaccination in patient 13, a DTH responder, but not in patient 11, a non-DTH responder (Fig. 2 A). Similarly, postvaccination induction of mesothelin-specific CD8⁺ T cells was also observed in the two other disease-free DTH responders (patient 8 [HLA-A2/A3] and patient 14 [HLA-A24]), but not in other non-DTH responders when tested with T2-A2/A3 (patient 2) and T2-A24 (patient 7) cells pulsed with the A2, A3 (Fig. 2 B), and A24 (Fig. 2 C) binding epitopes, respectively. A summary of the ELISPOT results evaluating vaccine-induced mesothelin-specific CD8⁺ T cell responses for all 14 patients treated with one allogeneic vaccination in this analysis is shown in Fig. 2 D. This difference in detection of mesothelin-specific T cell responses is statistically significant at a $P < 0.001$ by the Fisher exact test. These data suggest that there is a direct correlation between observed postvaccination in vivo DTH responses to autologous tumor and postvaccination in vivo mesothelin-specific CD8⁺ T cell responses for patients treated with an allogeneic vaccine in this paper. Specifically, each of the three DTH responders demonstrated a postvaccination induction in CD8⁺ T cell responses to two different mesothelin peptides that matched their respective HLA type, whereas only 1 out of 11 DTH nonresponders had an increased postvaccination mesothelin-specific CD8⁺ T cell response and only to a single peptide.

Poor Immune Status Does Not Explain the Failure to Measure Vaccine-induced, Mesothelin-specific CD8⁺ T Cell Responses in DTH Nonresponders. The data in Fig. 2 correlate in vivo DTH responses to autologous tumor with the postvaccination induction of mesothelin-specific CD8⁺ T cell responses. However, it is possible that this correlation represents generalized differences in overall immune function between the DTH responder and nonresponder patients, rather than a vaccine-specific induction of T cell responses to mesothelin in the DTH responder patients. To demonstrate that the postvaccination induction of mesothelin-specific CD8⁺ T cells is tumor antigen specific, we evaluated each HLA-A2⁺ patient for T cell responses to the HLA-A2 binding influenza matrix peptide, M1 (29). We chose the influenza M1 peptide because most patients on the vaccine analysis had received yearly influenza vaccines as a standard of care before enrollment. As shown in Fig. 3, all HLA-

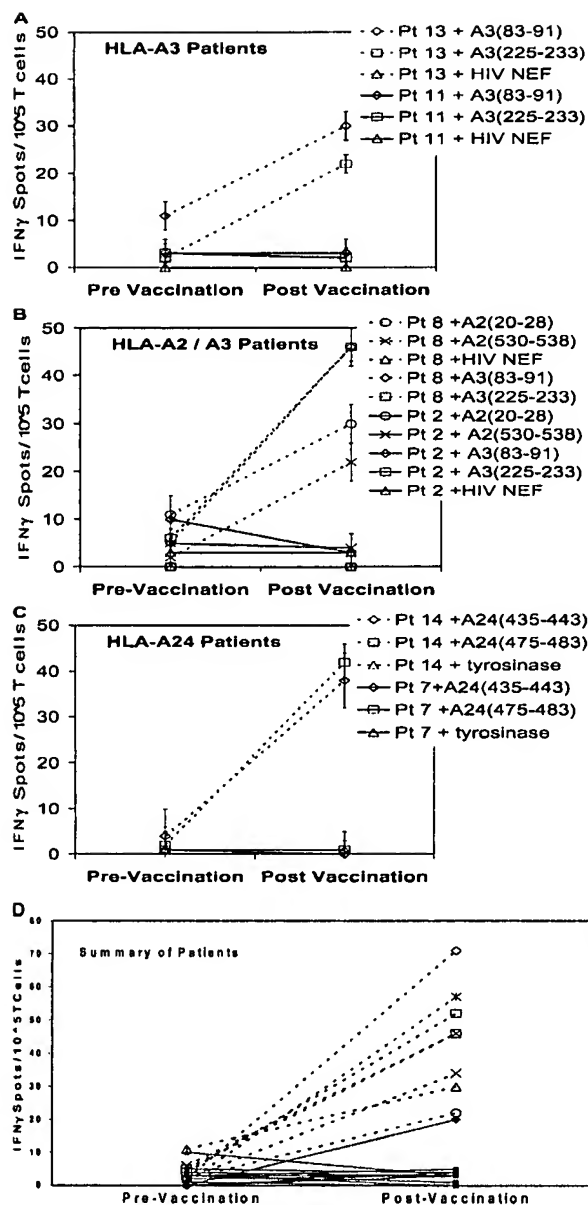


Figure 2. ELISPOT analysis of CD8⁺ T cells from PBMCs demonstrates postvaccination induction of mesothelin-specific T cells in three DTH responders. (A) ELISPOT analysis of PBLs from two patients who were HLA-A3⁺. (B) ELISPOT analysis of PBLs from two patients who were HLA-A2 and HLA-A3⁺. (C) ELISPOT analysis of PBLs from two patients who were HLA-A24⁺. (D) ELISPOT analysis of PBLs from all 14 patients who were treated with the vaccine (reference 24). ELISPOT analysis for IFN- γ -expressing cells was performed using PBMCs that were isolated on the day before vaccination or 28 d after the first vaccination. T2-A3 cells were pulsed with the two mesothelin-derived epitopes MesoA3₍₈₃₋₉₁₎ (squares), MesoA3₍₂₂₅₋₂₃₃₎ (X), and HIV-NEF₍₉₄₋₁₀₂₎ (not shown). T2-A2 cells were pulsed with the two mesothelin-derived epitopes MesoA2₍₂₀₋₂₈₎ (triangles), MesoA2₍₅₃₀₋₅₃₈₎ (circles), and HIV-gag₍₇₇₋₈₅₎ (not shown). T2-A24 cells were pulsed with the two mesothelin-derived epitopes MesoA24₍₄₃₅₋₄₄₃₎ (asterisks), MesoA24₍₄₇₅₋₄₈₃₎ (diamonds), and tyrosinase A24₍₂₀₆₋₂₁₄₎ (not shown). All DTH responders are represented by dotted lines and open symbols, and DTH nonresponders are represented by solid lines and closed symbols. For the detection of nonspecific background, the number of IFN- γ spots for CD8⁺ T cells spe-

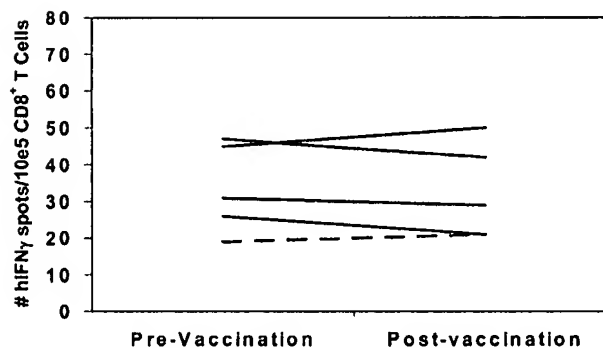


Figure 3. ELISPOT analysis of CD8⁺ T cells from PBMCs demonstrates similar pre- and postvaccination responses to the influenza matrix protein HLA-A2 binding epitope M1 (GILGFVFTL) in all HLA-A2⁺ patients. This analysis was performed on the same PBL samples described in Fig. 2. The DTH responders are represented by dotted lines, and the DTH nonresponders are represented by solid lines. For the detection of nonspecific background, the number of IFN- γ spots for CD8⁺ T cells specific for the irrelevant control peptides were counted. The HLA-A2 binding HIV-gag protein-derived epitope (SLYNTVATL), the HLA-A3 binding HIV-NEF protein-derived epitope (QVPLRPMTYK), and the HLA-A24 binding melanoma tyrosinase protein-derived epitope (AFLP-WHRLF) were used as negative control peptides in these assays. Data represent the mean of each condition assayed in triplicate, and standard deviations were <5%. The number of human IFN- γ spots per 10⁵ CD8⁺ T cells is plotted. Analysis of each patient's PBLs was performed at least twice, and all ELISPOT assays were performed in a blinded fashion.

A2⁺ patients demonstrated similar pre- and postvaccination T cell responses to the M1 peptide. Prevacination responses ranged from 19 to 50 IFN- γ spots per 10⁵ total CD8 T cells, and postvaccination responses remained approximately the same in each patient, unaffected by immunization with the pancreatic tumor vaccine (Fig. 3). A similar analysis confirmed that the HLA-A3 and HLA-A24 patients have detectable CD8⁺ T cell responses to influenza and EBV peptides (27, 28, 41), respectively, and that these responses are unaltered by immunization with the allogeneic pancreatic tumor vaccine (unpublished data).

In Vivo Cross-priming Explains the Induction of Mesothelin-specific CD8⁺ T Cells in Patients Vaccinated with the Allogeneic Pancreatic Tumor Vaccine. Vaccine-induced in vivo priming of host T cells by allogeneic tumor cells can occur by one of two mechanisms. The allogeneic tumor cells might directly prime the host's CD8⁺ T cells if the immunizing cells express HLA class I molecules in common with the host's HLA type. If not, transfer of the tumor antigen from the allogeneic cells to professional APCs would be required. Murine studies have already demonstrated that both

cific for the irrelevant control peptides were counted. The HLA-A2 binding HIV-gag protein-derived epitope (SLYNTVATL), the HLA-A3 binding HIV-NEF protein-derived epitope (QVPLRPMTYK), and the HLA-A24 binding tyrosinase protein-derived epitope (AFLPWHRLF) were used as negative control peptides in these assays. Background was minimal to negative control peptides, ranging from zero to three spots total (data included in each graph). Data represent the mean of each condition assayed in triplicate, and standard deviations were <5%. The number of human IFN- γ spots per 10⁵ CD8⁺ T cells is plotted. Analysis of each patient's PBLs was performed at least twice.

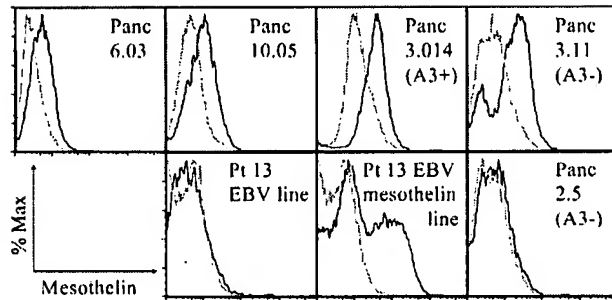


Figure 4. Expression of surface mesothelin of both the Panc 6.03 and Panc 10.05 vaccine and on cell lines used as targets for CTL assays. Panc 6.03, Panc 10.05, autologous EBV, autologous EBV transduced with mesothelin, Panc 2.5 (HLA-A3⁺), Panc 3.014 (HLA-A3⁺), and Panc 3.11 (HLA-A3⁻) were analyzed by flow cytometry for their levels of surface mesothelin using the mesothelin-specific monoclonal antibody CAK1 as the primary antibody and goat anti-mouse IgG1 FITC as the secondary antibody. The dotted line represents the isotype control, and the solid line represents mesothelin staining.

mechanisms can contribute to the induction of systemic antitumor immunity (42). However, controversy still remains as to whether vaccination can result in the induction of antigen-specific CD8⁺ T cells via the cross-priming mechanism that are efficient enough and in large enough quantities to treat actively growing cancer in patients. In this paper, we have evaluated the induction of HLA-A locus-restricted, mesothelin-specific CD8⁺ T cells in patients who received an allogeneic vaccine that is mismatched at the HLA-A locus (Table I). Specifically, we have demonstrated the induction of mesothelin-specific CD8⁺ T cells to HLA-A2, A3, and A24 mesothelin-derived peptides in patients receiving a mixture of two allogeneic pancreatic tumor vaccines. Both of these vaccine lines overexpress mesothelin (Fig. 4). However, neither vaccine line expresses HLA-A2, A3, or A24 (Table I). Therefore, these data provide direct evidence at the epitope level that allogeneic vaccine cells can activate CD8⁺ T cells against shared pancreatic tumor antigens, and that these CD8⁺ T cell responses are associated with other measures of in vivo immune responses. Because the vaccine cells are HLA mismatched with the three DTH responders' at the HLA-A locus, CD8⁺ T cell activation must occur by transfer of MHC class I antigens from the tumor cells to professional APCs, where they are processed and presented on MHC class I molecules via cross-priming.

Cross-Priming by Vaccine Recruited APCs Results in the Generation of Mesothelin-specific CD8⁺ T Cells Capable of Lysing Mesothelin-expressing Tumor Cells. The most important role of vaccine-induced CD8⁺ T cells in vivo is the ultimate lysis of antigen-expressing tumors. Currently, the best measure of this aspect of CD8⁺ T cell function is the ability of isolated CD8⁺ T cells to lyse antigen-expressing tumors in vitro. In an effort to correlate IFN- γ release with lytic activity in response to mesothelin, we analyzed the reactivity of a patient-derived T cell line to a panel of HLA-A3⁺ and HLA-A3⁻ tumor cell lines in a 4-h chromium release assay. The level of mesothelin expression of these tumor lines is shown in Fig. 4. Autologous DCs pulsed with the HLA-A3 mesothelin peptide 6293 were used to expand patient 13 CD8⁺ mesothelin-specific T cells in vitro. Cytolytic activity was assessed after three in vitro stimulations against HLA-A3⁺ and HLA-A3⁻ mesothelin-expressing target cells. As shown in Fig. 5 A, mesothelin-specific CD8⁺ T cells were able to lyse autologous EBV-transformed B cells transduced with the mesothelin gene, T2-A3 cells pulsed with the HLA-A3 mesothelin peptide 6293, and an HLA-A3⁺, mesothelin-expressing allogeneic tumor cell line, Panc 3.014. In a second study, lysis of the Panc 3.014 line could be blocked by the pan-HLA blocking antibody W6/32, but not by the isotype matched antibody against *S. mansoni* (unpublished data). In contrast, mesothelin-specific, patient-derived CD8⁺ T cells did not lyse well the mesothelin-expressing HLA-A3⁻ tumor cell line Panc 3.11, nor the HLA-A3-expressing, mesothelin-negative cell lines Panc 2.5 and the nonmesothelin-expressing autologous EBV-transformed B cells. After repeated in vitro stimulation, the T cell line lysed the Panc 3.014 mesothelin and HLA-A3-expressing line well (which was genetically modified to express HLA-A3), but not the original HLA-A3⁻, mesothelin-expressing Panc 3.014 tumor cell line (Fig. 5 B). These studies have been repeated six times with similar lysis results. These data confirm that an allogeneic vaccine can induce mesothelin-specific CD8⁺ T cell responses via the cross-priming mechanism, and that these CD8⁺ T cells are capable of lysing mesothelin-expressing cell line.

Discussion

These data describing CD8⁺ T cell responses induced by an allogeneic GM-CSF-secreting pancreatic tumor vac-

Table I. HLA Mismatch with the Vaccine Cells at the HLA-A Locus Provide Direct Human Evidence for MHC Class I Antigen Cross-Priming by APCs

	Vaccine line Panc 10.05	Vaccine line Panc 6.03	Patient 8	Patient 13	Patient 14
HLA class I expression at the A locus ^a	A1, A19	A1, A1	A2, A3	A3, A23	A1, A24

^aHLA typing was performed serologically and confirmed molecularly.

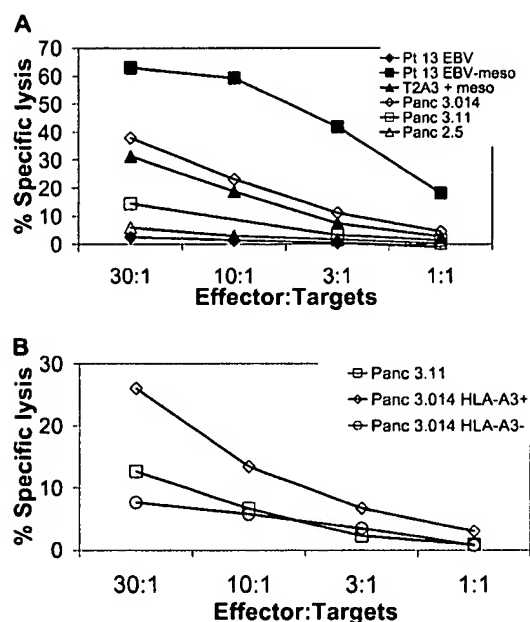


Figure 5. A mesothelin-specific CTL line derived from patient 13 PBL lyses HLA-A3⁺, mesothelin-expressing cells. Patient-derived CD8⁺ T cells stimulated with an HLA-A3 mesothelin peptide (peptide 6293) were tested for their capability to recognize and kill mesothelin-expressing tumor and EBV cell lines shown in Fig. 4. ⁵¹Cr-labeled target cells (3×10^3) were mixed with varying concentrations of patient 13 CD8⁺ T cell line (starting with 9×10^4) in a total of 200 μ l in a v-bottom 96-well plate. Percent lysis was calculated after 4 h at 37°C. Results are expressed as the percentage of specific lysis of triplicate samples.

cine support the following two conclusions. First, these findings provide direct human evidence that allogeneic vaccine cells induce CD8⁺ T cell responses by a cross-priming mechanism that requires transfer of antigen from the vaccine cells to professional APCs. Second, mesothelin is a new candidate pancreatic tumor antigen that can be used to analyze immune responses induced by whole cell pancreatic tumor vaccines.

The identification of shared, biologically relevant tumor antigens provides the opportunity to study the mechanisms by which vaccines induce antitumor immune responses. An important result of this paper is the direct demonstration of cross-priming at the MHC class I epitope level. Controversy still exists as to whether cross-priming is a clinically important mechanism for in vivo priming of CD8⁺ T cells (18). Previously published murine studies evaluating whole cell vaccines have shown that the professional APCs of the host can prime both CD4⁺ and CD8⁺ T cells, both of which are required for generating systemic antitumor immunity (14, 22, 23). Furthermore, Jung et al. have previously shown that cross-priming is relevant in vivo because depletion of CD11C⁺ DC abrogated effective immunization in mice (43). Cross-priming has also been shown to play an important role in generating CD8⁺ T cell responses to infectious diseases (44–46). Furthermore, human tumor studies have demonstrated that both macro-

phages and DCs can take up antigens in vitro and prime naive CD8⁺ T cells by the cross-priming mechanism (5, 8, 11–16, 44–46). However, other studies suggest that the mechanism of cross-priming is inefficient at inducing CD8⁺ T cell responses in vivo in vaccinated healthy subjects (47). Several factors may explain the differences in results between these studies. First, the efficiency of cross-priming in vivo may be influenced by several factors, including the following: the route of APC exposure to the antigen (44), whether the APC is a macrophage or DC (6, 17, 47), the maturation status of the APCs (48), and the form of antigen taken up by the APCs (4, 11). The form of the antigen that is presented to the APCs has been of particular interest because several recent studies suggest that apoptotic tumors are more efficiently processed and presented by an APC than necrotic tumor or soluble protein (8, 11). Furthermore, we have reported previously that irradiated GM-CSF-secreting vaccine cells are much more efficient at inducing systemic immune responses than unirradiated vaccine cells or vaccine cells that are inactivated by nonapoptotic inducing mechanisms (49). In addition, several studies have used the ovalbumin antigen system, which is a strong foreign antigen that may be processed and presented differently than naturally occurring tumor-associated antigens (4, 6).

The biologic importance of these data in demonstrating CD8⁺ T cell cross-priming is strongly supported by our data correlating these responses with in vivo postvaccination DTH responses to autologous tumor cells. Until now, observed postvaccination DTH responses against autologous tumor cells has provided the best evidence in support of a vaccine-induced, T cell-mediated antitumor immunity in patients treated in clinical trials (24, 50–57). Three recent papers have linked antibody responses to clinical responses in patients receiving a melanoma vaccine (58, 59) and a human chorionic gonadotropin-based vaccine (60). However, this paper also demonstrates postvaccination in vitro antigen-specific T cell responses that correlate with in vivo evidence of immune induction (DTH responses to autologous tumor cells). In addition, the use of uncultured lymphocytes rather than T cell lines and clones that have been in long-term culture demonstrates immune responses that are more closely associated with human in vivo T cell function. Unfortunately, it is difficult to demonstrate direct pancreatic cell killing without several rounds of in vitro CD8⁺ T cell expansion. However, after three rounds of stimulation with autologous DCs pulsed with the A3 mesothelin peptide, these T cells can lyse a pancreatic tumor line and other mesothelin-expressing cell lines, providing evidence that mesothelin can serve as a tumor rejection target of T cells. Only a small panel of tumor lines were tested due to the significant challenge in generating in vitro pancreatic tumor lines, including the three DTH responder patients. However, the fact that the T cells lyse mesothelin-expressing HLA-A3⁺ tumor cells, but not mesothelin-expressing HLA-A3[−] lines, demonstrates the mesothelin and HLA-restricted specificity of the T cell activity.

In this paper, we also demonstrate that mesothelin-specific T cells can be induced against at least six different peptides presented by three different HLA-A locus alleles. T cell responses determined by ELISPOT were comparable for each of the epitopes. This finding provides further support that mesothelin can serve as a shared antigen. This finding also provides further evidence that cross-priming is an efficient mechanism for antigen processing and presentation onto MHC class I. It is interesting to point out that the highest-ranking antigenic epitopes predicted to be the best HLA-A allele binding epitopes based on their motif, bound to their respective HLA alleles and were also recognized by mesothelin-specific T cells. Papers analyzing other tumor antigens have found that the highest ranking epitopes do not necessarily correlate with optimal recognition by T cells (35). We also performed the computer algorithms on two melanoma antigens, tyrosinase and MAGE 1, to determine how their published HLA-A2 binding peptides rank by this method (61, 62). We found that our HLA-A2 binding mesothelin epitopes were given similar scores as the known tyrosinase and MAGE 1 HLA-A2 binding epitopes. This was also true for the published HLA-A2 HIV-gag and HLA-A3 HIV-NEF epitopes that were used as control antigens in our analyses (30, 31). Choosing epitopes that rank high by both algorithms appears to be an important predictor of the probability of binding to the respective HLA molecule. However, the likelihood of successfully predicting HLA binding epitopes will probably be determined in part by the antigen being studied. Studies aimed at addressing this question are underway.

In conclusion, we have directly demonstrated cross-priming at the MHC class I epitope level. These studies were facilitated through the development of a functional genomic approach that identified mesothelin as a new candidate pancreatic tumor antigen recognized by CD8⁺ T cells. The correlation of in vitro T cell responses with in vivo measures of immunologic response validates the biologic importance of this approach. Because we detected evidence for cross presentation in the three patients with a better clinical course, we suspect that cross-presentation may have clinical relevance, but larger studies are required to investigate this.

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EXHIBIT 3

BIOLOGICAL THERAPEUTIC AGENTS

Immunological Targets

C28 A Safety and Efficacy Trial of Lethally Irradiated Allogeneic Pancreatic Tumor Cells Transfected with the GM-CSF Gene in Combination

with Adjuvant Chemoradiotherapy for the Treatment of Adenocarcinoma

of the Pancreas. Dan Laheru,¹ Charles Yeo,¹ Barb Biedrzycki,¹ Beth Onners,¹ Irena Tartakovsky,¹ Sara Solt,¹ Ralph Hruban,¹ Keith Lillemoe,² John Cameron,¹ Ross Abrams,³ Elizabeth Garrett-Mayer,¹ Elizabeth Jaffee,¹ Sidney Kimmel Comprehensive

Cancer Ctr.,¹ Baltimore, MD, Indiana University School of Medicine,²

Indianapolis, Indiana, Rush University School of Medicine,³ Chicago, Illinois.

Background: Pancreatic cancer remains the fourth leading cause of cancer related deaths in the US in 2005 ¹. Surgical resection provides the only possibility of cure. However, the historical five-year survival remains approximately 15-20% with one and two year survival of 63% and 42% respectively ². Recently the Virginia Mason Medical Center published a study of 53 patients with resected pancreas cancer treated with 5-Fluorouracil (5-FU) administered continuous infusion (CI) with weekly cisplatin and every other day interferon-alpha with radiotherapy. The clinical efficacy is encouraging but with significant treatment-related side effects ³. In contrast, a randomized European trial in patients with resected pancreatic cancer demonstrated a survival benefit in the chemotherapy alone arm ⁴. As such, a standard adjuvant treatment approach for patients with resected disease has not yet been determined. We have developed an irradiated GM-CSF transfected allogeneic whole cell line pancreas adenocarcinoma vaccine and previously reported the results of this vaccine administered intradermally (ID) in sequence with chemoradiotherapy in 14 patients with resected pancreatic adenocarcinoma ⁵. We report a follow-up 60 patient study in this same population using the highest bioactive vaccine dose identified in the initial phase I study. Methods: Single institution phase II study of 60 patients with resected pancreatic adenocarcinoma administered a total of 5 vaccines using two pancreatic cancer cell lines each delivering 2.5×10^8 cells ID. Vaccine one was administered 8-10 weeks following surgical resection. Patients subsequently were treated with 5-FU CI based chemotherapy integrated with radiotherapy. Patients who were disease-free one month after completion of chemoradiotherapy received vaccines 2-4, each 1 month apart. A fifth and final booster vaccine was administered 6 months after vaccine 4. The objectives of the study were: 1.To estimate overall survival and disease-free survival in patients with minimal residual disease treated with adjuvant chemoradiotherapy in sequence with the irradiated allogeneic GM-CSF transfected pancreatic tumor cell lines versus chemoradiotherapy alone. 2.To estimate the association of specific *in -vivo* parameters of immune response with clinical responses in patients with

minimal residual disease treated with combination chemoradiotherapy together with the irradiated allogeneic GM-CSF transfected pancreatic tumor cell lines. The specific immune parameters include: post-vaccination delayed type hypersensitivity reactions to autologous tumor, the degree of local eosinophil, macrophage, and T cell infiltration at the vaccine site, and mesothelin-specific T cell responses. 3. To further identify and characterize toxicities associated with intradermal injections of the vaccine that were initially reported in the phase 1 trial. Results/Conclusions: The study completed enrollment of new patients in January 2005. At this early analysis, 56 patients are evaluable at one year and 36 patients are evaluable at two years. Median follow-up for these patients is approximately 32 months. 1. The administration of a GM-CSF allogeneic pancreas cancer vaccine is safe and well tolerated. Treatment related side effects included transient vaccine injection site reactions. 2. Systemic GM-CSF levels were evaluated as an indirect measure of the longevity of vaccine cells at the immunizing site. As was observed in the phase I study, GM-CSF levels peaked at 48 hours following the 1st and 2nd vaccination, but peaked earlier following the 3rd and 4th vaccination with diminution in amplitude. Serum GM-CSF levels following vaccine 5 peaked again at 48 hours but returned to vaccine 1 serum levels. 3. At this early analysis, the one and two year survival are 88% and 76% respectively. While the data is only preliminary, this study compares very favorably with the available published data. 4. Immune correlates will be presented at a future date.

EXHIBIT 4



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Assessing prognosis in metastatic pancreatic cancer by the serum tumor marker CA 19-9: pretreatment levels or kinetics during chemotherapy?

Boeck S, Schulz C, Stieber P, Holdenrieder S, Weckbach S, Heinemann V.

Department of Internal Medicine III, Klinikum Grosshadern, Ludwig Maximilians University of Munich, Germany. stefan.boeck@med.uni-muenchen.de

BACKGROUND: The carbohydrate antigen 19-9 (CA 19-9) is currently the most widely used serum tumor marker in pancreatic cancer (PC). CA 19-9 pretreatment levels as well as CA 19-9 kinetics during systemic chemotherapy can provide prognostic information regarding survival of patients with metastatic PC. **CASE REPORTS:** We report the clinical course of 2 patients with metastatic PC who underwent palliative chemotherapy with gemcitabine. Both patients showed a significant elevation of pretreatment CA 19-9 levels (7,505 and 150,000 U/ml, respectively), however, subsequently they experienced a highly significant reduction (>90%) of CA 19-9 kinetics under gemcitabine chemotherapy. A good disease control and a clinical benefit response were achieved in both patients. Time to tumor progression was 30 weeks and 28 weeks, overall survival 14 months and 11 months, respectively. **CONCLUSION:** These data indicate that CA 19-9 kinetics under chemotherapy may possibly serve as a useful surrogate marker for time to tumor progression and survival in advanced PC.

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Irinotecan plus gemcitabine induces both radiographic and CA 19-9 tumor marker responses in patients with previously untreated advanced pancreatic cancer. [J Clin Oncol. 2002]

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CA 19-9 in evaluating the response to chemotherapy in advanced pancreatic cancer. [Hepato-gastroenterology. 1997]

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EXHIBIT 5



US 20050249748A1

(19) **United States**(12) **Patent Application Publication****Dubensky, JR. et al.**(10) **Pub. No.: US 2005/0249748 A1**(43) **Pub. Date: Nov. 10, 2005**(54) **RECOMBINANT NUCLEIC ACID MOLECULES, EXPRESSION CASSETTES, AND BACTERIA, AND METHODS OF USE THEREOF**(76) **Inventors:** Thomas W. Dubensky JR., Piedmont, CA (US); Daniel A. Portnoy, Albany, CA (US); William S. Luckett JR., Richmond, CA (US); David N. Cook, Lafayette, CA (US)**Correspondence Address:**
MORRISON & FOERSTER LLP
755 PAGE MILL RD
PALO ALTO, CA 94304-1018 (US)(21) **Appl. No.: 11/021,441**(22) **Filed: Dec. 23, 2004****Related U.S. Application Data**

(63) Continuation-in-part of application No. PCT/US04/23881, filed on Jul. 23, 2004, which is a continuation-in-part of application No. 10/883,599, filed on Jun. 30, 2004, and which is a continuation-in-part of application No. 10/773,618, filed on Feb. 6, 2004. Said application No. 10/883,599 is a continuation-in-part of application No. 10/773,792, filed on Feb. 6, 2004, and which is a continuation-in-part of application No. 10/773,618, filed on Feb. 6, 2004. Said application No. PCT/US04/23881 is a continuation-in-part of application No. 10/773,792, filed on Feb. 6, 2004.

Continuation-in-part of application No. 10/883,599, filed on Jun. 30, 2004, and which is a continuation-in-part of application No. 10/773,618, filed on Feb. 6, 2004.

Said application No. 10/883,599 is a continuation-in-part of application No. 10/773,792, filed on Feb. 6, 2004.

Continuation-in-part of application No. 10/773,618, filed on Feb. 6, 2004.

Continuation-in-part of application No. 10/773,792, filed on Feb. 6, 2004.

(60) Provisional application No. 60/616,750, filed on Oct. 6, 2004. Provisional application No. 60/615,287, filed on Oct. 1, 2004. Provisional application No. 60/599,377, filed on Aug. 5, 2004. Provisional application No. 60/556,744, filed on Mar. 26, 2004. Provisional

application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/532,598, filed on Dec. 24, 2003. Provisional application No. 60/556,744, filed on Mar. 26, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/532,598, filed on Dec. 24, 2003. Provisional application No. 60/556,744, filed on Mar. 26, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/532,598, filed on Dec. 24, 2003. Provisional application No. 60/556,744, filed on Mar. 26, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/532,598, filed on Dec. 24, 2003. Provisional application No. 60/541,515, filed on Feb. 2, 2004. Provisional application No. 60/541,515, filed on Feb. 2, 2004.

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(52) **U.S. Cl.** **424/190.1; 435/69.3; 435/471; 435/252.3; 530/350; 536/23.7**(57) **ABSTRACT**

The present invention provides recombinant nucleic acid molecules, expression cassettes, and vectors useful for expression of polypeptides, including heterologous polypeptides, such as antigens, in bacteria. Some of the recombinant nucleic acid molecules, expression cassettes and vectors comprise codon-optimized sequences encoding the polypeptides and/or signal peptides. Some of the recombinant nucleic acid molecules, expression cassettes, and expression vectors comprise sequences encoding non-Listerial and/or non-secA1 signal peptides for secretion of the polypeptides. The invention also provides bacteria comprising the nucleic acid molecules, expression cassettes, and expression vectors, as well as compositions such as vaccines comprising the bacteria. Methods of making and using the bacteria, recombinant nucleic acid molecules, and expression cassettes are also provided.

Listeria hly DP-L4056 and EGD Alignment

Query: Listeria EGD
Subject: DP-L4056 (wild-type, Portnoy strain)

Query: 1 ggtacctccttgattagtagtatattccctatctttaaagtgactttttatgttgaggcataaac 60
|||||
Sbjct: 1 ggtacctccttgattagtagtatattccctatctttaaagtgtactttttatgttgaggcataaac 60

prfA Box

Query: 61 atttggttaacgacgataaaaggacagcaggactagaataaaagctataaaagcaagcatata 120
|||||
Sbjct: 61 atttggttaatgacgtcaaaaggatagcaagactagaataaaagctataaaagcaagcatata 120

Query: 121 atattgcgttttcacatcttttagaagcggaatttcgcccaatattataattatcaaaagagaggg 180
|||||
Sbjct: 121 atattgcgttttcacatcttttagaagcggaatttcgcccaatattataattatcaaaagagaggg 180

Shine-Dalgarno LLO start
Query: 181 gtggcaaacgggtatttggcattatttaggttaaaaaatgtagaaggagagtgaaccatg 240
|||||
Sbjct: 181 gtggcaaacgggtatttggcattatttaggttaaaaaatgtagaaggagagtgaaccatg 240

FIGURE 1

Construct: LLO_{ss}-PEST-hEphA2

Native LLO signal peptide + PEST fused to full-length human EphA2

Not Codon optimized

No epitope tags (e.g., myc or FLAG used in this construct)

Fusion protein coding sequence shown

```
ATGAAAAAATAATGCTAGTTTTTATTACACTTATATTAGTTAGTCTACCAATTGCGCAACAAACTGAA
GCAAAGGATGCATCTGCATTCAATAAAGAAAATTCAATTTTCATCCATGGCACCACCAGCATCTCCGCC
TGCAAGTCTAAGACGCCAATCGAAAAGAAAACACGCGGATCTCGAGCTCCAGGCAGCCCGCGCCTGC
TTCGCCCTGCTGTGGGGCTGTGCGCTGGCCGCGGCGCGGCGGCGCAGGGCAAGGAAGTGGTACTGCT
GGACTTTGCTGCACTGGAGGGGAGCTCGGCTGGCTCACACACCCGTATGGCAAAGGGTGGGACCTG
ATGCAGAACATCATGAATGACATGCCGATCTACATGTAATCCGTGTGCAACGTGATGTCTGGCGACCA
GGACAACCTGGCTCCGCACCAACTGGGTGTACCGAGGAGAGGCTGAGCGTATCTTCATTGAGCTCAAGT
TTACTGTACGTGACTGCAACAGCTTCCCTGGTGGCGCCAGCTCCTGCAAGGAGACTTTCAACCTCTACT
ATGCCGAGTCGGACCTGGACTACGGCACCAACTTCAGAGAAGCGCTGTTCACCAAGATTGACACCATT
GCGCCCGATGAGATCACCCTCAGCAGCGAATTGAGGACGCGCAGCCACGTGAAGCTGAACGTGGAGGAGC
GCTCCGTGGGGCCGCTCACC CGCAAAGGCTTCTACCTGGCCTTCCAGGATATCGGTGCCTGTGTGGCG
CTGCTCTCCGTCCGTGTCTACTACAAGAAGTGCCCGAGCTGCTGCAGGGCCTGGCCCACTTCCCTGAG
ACCATCGCCGCTCTGATGCACCTTCCCTGGCCACTGTGGCCGGCACCTGTGTGGACCATGCCGTGGTG
CCACCGGGGGTGAAGAGCCCGTATGCACTGTGCACTGGATGGCGAGTGGCTGGTGCCCATTTGGGC
AGTGCCTGTGCCAGGCAGGCTACGAGAAGGTGGAGGATGCCTGCCAGGCCTGCTCGCCTGGATTTTTT
AAGTTTGAGGCATCTGAGAGCCCCTGCTTGGAGTGCCCTGAGCACACGCTGCCATCCCCTGAGGGTGC
CACCTCTGCGAGTGTGAGGAAGGCTTCTCCGGGCACCTCAGGACCCAGCGTCGATGCCTTGACAC
GACCCCCCTCCGCCCCACACTACCTCACAGCCGTGGGCATGGGTGCCAAGGTGGAGCTGCGCTGGACG
CCCCCTCAGGACAGCGGGGGCCGCGAGGACATTGTCTACAGCGTCACTGCGAACAGTGTCTGGCCCGA
GTCTGGGGAATGCGGGCCGTGTGAGGCCAGTGTGCGCTACTCGGAGCCTCCTACGGACTGACCCGCA
CCAGTGTGACAGTGAGCGACCTGGAGCCCCACATGAACTACACCTTCACCGTGGAGGGCCGCAATGGC
GTCTCAGGCCTGGTAACCAGCCGAGCTTCCGTACTGCCAGTGTGACGATCAACCAGACAGAGCCCC
CAAGGTGAGGCTGGAGGGCCGAGCACCACTCGCTTAGCGTCTCCTGGAGCATCCCCCGCCGAGC
AGAGCCGAGTGTGGAAGTACGAGGTCACTTACCGCAAGAAGGGAGACTCCAACAGCTACAATGTGCG
CCGACCGAGGGTTTCTCCGTGACCTGGACGACCTGGCCCGAGACACCACCTACCTGGTCCAGGTGC
AGGCACTGACGAGGAGGGCCAGGGGGCCGCGAGCAGGGTGCACGAATTCAGACGCTGTCCCCGGA
GGGATCTGGCAACTTGGCGGTGATTGGCGGCGTGGCTGTGGTGTGGTCTGCTTCTGGTGTGGCAG
GAGTTGGCTTCTTTATCCACCGCAGGAGGAAGAACCAGCGTGCCCGCCAGTCCCCGGAGGACGTTTAC
TTCTCCAAGTCAGAACAACTGAAGCCCCCTGAAGACATACGTGGACCCCCACACATATGAGGACCCCA
CCAGGCTGTGTTGAAGTTCACTACCGAGATCCATCCATCCTGTGTCACTCGGCAGAAAGGTGATCGGAG
CAGGAGAGTTTGGGGAGGTGTACAAGGGCATGCTGAAGACATCCTCGGGGAAGAAGGAGGTGCCGGT
GGCCATCAAGACGCTGAAAGCCGGCTACACAGAGAAGCAGCGAGTGGACTTCTCGGCGAGGCCGGC
ATCATGGGCCAGTTCAGCCACCACAACATCATCCGCCCTAGAGGGCGTCATCTCCAAATACAAGCCCAT
GATGATCATCACTGAGTACATGGAGAATGGGGCCCTGGACAAGTTCTTCCGGGAGAAGGATGGCGAG
TTCAGCGTGCTGCAGCTGGTGGGCATGCTGCGGGGCATCGCAGCTGGCATGAAGTACCTGGCCAACAT
GAACTATGTGCACCGTGACCTGGCTGCCCGCAACATCCTCGTCAACAGCAACCTGGTCTGCAAGGTGT
CTGACTTTGGCCTGTCCCGCTGCTGGAGGACGACCCGAGGCCACCTACACCACCACTGGCGGCAAG
ATCCCCATCCGCTGGACCGCCCGGAGGCCATTTCTACCGGAAGTTCACCTCTGCCAGCGACGTGTG
GAGCTTTGGCATTGTGATGTGGGAGGTGATGACCTATGGCGAGCGGCCCTACTGGGAGTTGTCCAACC
ACGAGGTGATGAAAGCCATCAATGATGGCTTCCGGCTCCCCACACCCATGGACTGCCCTCCGCCATC
TACCAGCTCATGATGCACTGCTGGCAGCAGGAGCGTGCCCGCCGCCCAAGTTCGCTGACATCGTCAG
CATCTGGACAAGCTCATTGCTGCCCTGACTCCCTCAAGACCTGGCTGACTTTGACCCCCGCGTGTG
TATCCGGCTCCCCAGCACGAGCGGCTCGGAGGGGGTGGCCTTCCGCACGGTGTCCGAGTGGCTGGAGT
CCATCAAGATGCAAGCAGTATACGGAGCACTTCAATGGCGGCCGGCTACACTGCCATCGAGAAGGTGGTG
CAGATGACCAACGACGACATCAAGAGGATTGGGGTGGCGCTGCCCGGCCACCAAGCGCATCGCCT
ACAGCCTGTGGGACTCAAGGACCAGGTGAACACTGTGGGGATCCCCATC
```

FIGURE 2

Construct: LLOss-PEST-hEphA2

Native LLO signal peptide + PEST fused to full-length human EphA2

Not Codon optimized

No epitope tags (e.g., myc or FLAG used in this construct)

Predicted fusion protein shown

MKKIMLVFITLILVSLPIAQQTAKDASAFNKEN
SISSMAPPASPPASPKTPIEKKHADLELQAARAC
FALLWGCALAAAAAQAQKEVVLLDFAAAGGELG
WLTHPYGKGWDLMQNIMNDMPIYMYSVCNVMS
GDQDNWLRTNWVYRGEAERIFIELKFTVRDCNSF
PGGASSCKETFNLYYAESDLDYGTNFQKRLFTKI
DTIAPDEITVSSDFEARHVKLNVEERSVGPLTRK
GEYLAFAQDIGACVALLSVRVYYKKCPPELLQGLA
HFPETIAGSDAPSLATVAGTCVDHAVVPPGGEEP
RMHCAVDGEWLVPIGQCLCQAGYEKVEDACQAC
SPGFFKFEEASESPCLECPEHTLPSPEGATSCECEE
GFFRAPQDPASMPCTRPPSAPHYLTAVGMGAKV
ELRWTPPQDSGGREDIVYSVTCEQCWPESGECGP
CEASVRYSEPPHGLTRTSVTVSDLEPHMNYTFTV
EARNGVSGLVTSRSFRTASVSINQTEPPKVRLEG
RSTTSLSVSWSSIPPPQQSRVWKYEVTYRKKGDSN
SYNVRRTTEGFSVTLLDDLAPDTTYLVQVQALTQE
GQGAGSRVHEFQTLSPEGSGNLAVIGGVAVGVV
LLLVLAVGVGFFIHRRRKKNQRARQSPEDVYFSKSE
QLKPLKTYVDPHTYEDPNQAVLKFTTEIHPSCVT
RQKVIGAGEFGEVYKGMMLKTSSGKKEVPVAIKTL
KAGYTEKQQRVDFLGEAGIMGQFSHHNIIRLEGVI
SKYKPMMIITEYMEENGALDKFLREKDGESVLQL
VGMLRGIAAGMKYLANMNYVHRDLAARNILVNS
NLVCKVSDFGLSRVLEDDPEATYTTSGGKIPIRW
TAPEAISYRKFTSASDVWSFGIVMWEVMTYGERP
YWELSNHEVMKAINDGFRLPTPMDCPSAIYQLM
MQCWQQERARRPKFADIVSILDKLIRAPDSLKTL
ADFDPRVSIRLPSTSGSEGVPFRTVSEWLESIKM
QQYTEHFMAAGYTAIEKVVQMTNDDIKRIGVRL
PGHQKRIAYSLLGLKDQVNTVGIP

FIGURE 3

EphA2 EX2 domain
Native nucleotide sequence

CAGGGCAAGGAAGTGGTACTGCTGGACTTTGCTGCAGCTGGAGGGGAGCTCGGCTG
GCTCACACACCCGTATGGCAAAGGGTGGGACCTGATGCAGAACATCATGAATGACA
TGCCGATCTACATGTACTCCGTGTGCAACGTGATGTCTGGCGACCAGGACAACTGGC
TCCGCACCAACTGGGTGTACCGAGGAGAGGCTGAGCGTATCTTCATTGAGCTCAAGT
TTACTGTACGTGACTGCAACAGCTTCCCTGGTGGCGCCAGCTCCTGCAAGGAGACTT
TCAACCTCTACTATGCCGAGTCGGACCTGGACTACGGCACCAACTTCCAGAAGCGCC
TGTTACCAAGATTGACACCATTGCGCCCGATGAGATCACCGTCAGCAGCGACTTCG
AGGCACGCCACGTGAAGCTGAACGTGGAGGAGCGCTCCGTGGGGCCGCTCACCCGC
AAAGGCTTCTACCTGGCCTTCCAGGATATCGGTGCCTGTGTGGCGCTGCTCTCCGTC
CGTGTCTACTACAAGAAGTGCCCCGAGCTGCTGCAGGGCCTGGCCCCACTTCCCTGAG
ACCATCGCCGGCTCTGATGCACCTTCCCTGGCCACTGTGGCCGGCACCTGTGTGGAC
CATGCCGTGGTGCCACCGGGGGGTGAAGAGCCCCGTATGCACTGTGCAGTGGATGG
CGAGTGGCTGGTGCCCATTTGGGCAGTGCCTGTGCCAGGCAGGCTACGAGAAGGTGG
AGGATGCCTGCCAGGCCTGCTCGCCTGGATTTTTTAAGTTTGAGGCATCTGAGAGCC
CCTGCTTGAGTGCCCTGAGCACACGCTGCCATCCCCTGAGGGTGCCACCTCCTGCG
AGTGTGAGGAAGGCTTCTTCCGGGCACCTCAGGACCCAGCGTCGATGCCTTGACAC
GACCCCCCTCCGCCCCACACTACCTCACAGCCGTGGGCATGGGTGCCAAGGTGGAG
CTGCGCTGGACGCCCCCTCAGGACAGCGGGGGCCGCGAGGACATTGTCTACAGCGT
CACCTGCGAACAGTGCTGGCCGAGTCTGGGGAATGCGGGCCGTGTGAGGCCAGTG
TGCGCTACTCGGAGCCTCCTCACGGACTGACCCGCACCAAGTGTGACAGTGAGCGAC
CTGGAGCCCCACATGAACTACACCTTACCCTGGAGGGCCCGCAATGGCGTCTCAGG
CCTGGTAACCAGCCGCAGCTTCCGTACTGCCAGTGTGAGCATCAACCAGACAGAGC
CCCCAAGGTGAGGCTGGAGGGCCGCAGCACCACTCGCTTAGCGTCTCCTGGAGC
ATCCCCCGCCGCAGCAGAGCCGAGTGTGGAAGTACGAGGTCACTTACCGCAAGAA
GGGAGACTCCAACAGCTACAATGTGCGCCGCACCGAGGGTTTCTCCGTGACCCTGG
ACGACCTGGCCCCAGACACCACCTACCTGGTCCAGGTGCAGGCACTGACGCAGGAG
GGCCAGGGGGCCGGCAGCAGGGTGCACGAATTCCAGACG

FIGURE 4

EphA2 EX2 domain
Nucleotide sequence for optimal codon usage in *Listeria*

CAAGGTAAAGAAGTTGTTTTATTAGATTTTGCAGCAGCAGGTGGTGAATTAGGTTGG
TTAACACATCCATATGGTAAAGGTTGGGATTTAATGCAAAATATTATGAATGATATG
CCAATTTATATGTATAGTGTTTGTAATGTTATGAGTGGTGATCAAGATAATTGGTTAC
GTACAAATTGGGTTTATCGTGGTGAAGCAGAACGTATTTTATTGAATTAAATTTA
CAGTTCGTGATTGTAATAGTTTTCCAGGTGGTGCAGTAGTTGTAAAGAAACATTTA
ATTTATATTATGCAGAAAGTGATTTAGATTATGGTACAAATTTTCAAAAACGTTTATT
TACAAAAATTGATACAATTGCACCAGATGAAATTACAGTTAGTAGTGATTTTGAAGC
ACGTCATGTTAAATTAATGTTGAAGAACGTAGTGTTGGTCCATTAACACGTAAAGG
TTTTTATTTAGCATTTCAAGATATTGGTGCATGTGTTGCATTATTAAGTGTTTCGTGTTT
ATTATAAAAAATGTCCAGAATTATTACAAGGTTTAGCACATTTTCCAGAAACAATTG
CAGGTAGTGATGCACCAAGTTTAGCAACAGTTGCAGGTACATGTGTTGATCATGCAG
TTGTTCCACCAGGTGGTGAAGAACCACGTATGCATTGTGCAGTTGATGGTGAATGGT
TAGTTCCAATTGGTCAATGTTTATGTCAAGCAGGTTATGAAAAAGTTGAAGATGCAT
GTCAAGCATGTAGTCCAGGTTTTTTTTAAATTTGAAGCAAGTGAAAGTCCATGTTTAG
AATGTCCAGAACATACATTACCAAGTCCAGAAGGTGCAACAAGTTGTGAATGTGAA
GAAGGTTTTTTTCGTGCACCACAAGATCCAGCAAGTATGCCATGTACACGTCCACCA
AGTGCACCACATTATTTAACAGCAGTTGGTATGGGTGCAAAAGTTGAATTACGTTGG
ACACCACCACAAGATAGTGGTGGTCGTGAAGATATTGTTTATAGTGTTACATGTGAA
CAATGTTGGCCAGAAAGTGGTGAATGTGGTCCATGTGAAGCAAGTGTTTCGTTATAGT
GAACCACCACATGGTTTAACACGTACAAGTGTTACAGTTAGTGATTTAGAACCACAT
ATGAATTATACATTTACAGTTGAAGCACGTAATGGTGTTAGTGGTTTAGTTACAAGT
CGTAGTTTTTCGTACAGCAAGTGTTAGTATTAATCAAACAGAACCACCAAAAAGTTCGT
TTAGAAGGTTCGTAGTACAACAAGTTTAAGTGTTAGTTGGAGTATCCACCACCACAA
CAAAGTCGTGTTTGGAAATATGAAGTTACATATCGTAAAAAAGGTGATAGTAATAG
TTATAATGTTTCGTTCGTACAGAAGGTTTTAGTGTTACATTAGATGATTTAGCACCAGA
TACAACATATTTAGTTCAAGTTCAAGCATTAACACAAGAAGGTCAAGGTGCAGGTA
GTCGTGTTTCATGAATTTCAAACA

FIGURE 5

EphA2 EX2 domain
Primary Amino Acid Sequence

Q G K E V V L L D F A A A G G E L G W L T H P Y G K G W D L M Q
N I M N D M P I Y M Y S V C N V M S G D Q D N W L R T N W V Y R
G E A E R I F I E L K F T V R D C N S F P G G A S S C K E T F N L Y
Y A E S D L D Y G T N F Q K R L F T K I D T I A P D E I T V S S D F E
A R H V K L N V E E R S V G P L T R K G F Y L A F Q D I G A C V A
L L S V R V Y Y K K C P E L L Q G L A H F P E T I A G S D A P S L A
T V A G T C V D H A V V P P G G E E P R M H C A V D G E W L V P I
G Q C L C Q A G Y E K V E D A C Q A C S P G F F K F E A S E S P C L
E C P E H T L P S P E G A T S C E C E E G F F R A P Q D P A S M P C
T R P P S A P H Y L T A V G M G A K V E L R W T P P Q D S G G R E
D I V Y S V T C E Q C W P E S G E C G P C E A S V R Y S E P P H G L
T R T S V T V S D L E P H M N Y T F T V E A R N G V S G L V T S R S
F R T A S V S I N Q T E P P K V R L E G R S T T S L S V S W S I P P P
Q Q S R V W K Y E V T Y R K K G D S N S Y N V R R T E G F S V T L
D D L A P D T T Y L V Q V Q A L T Q E G Q G A G S R V H E F Q T

FIGURE 6

Construct: LLOss-PEST-EX2_hEphA2

Native LLO signal peptide + PEST fused to external domain of human EphA2

Not Codon optimized

No epitope tags (e.g., myc or FLAG used in this construct)

Fusion protein coding sequence shown

ATGAAAAAATAATGCTAGTTTTTATTACACTTATATTAGTTAGTCTACCAATTGCGC
AACAAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAATTCAATTTTCATCC
ATGGCACCACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGAAAAGAAACA
CGCGGATCTCGAGCAGGGCAAGGAAGTGGTACTGCTGGACTTTGCTGCAGCTGGAG
GGGAGCTCGGCTGGCTCACACACCCGTATGGCAAAGGGTGGGACCTGATGCAGAAC
ATCATGAATGACATGCCGATCTACATGTACTCCGTGTGCAACGTGATGTCTGGCGAC
CAGGACAACCTGGCTCCGCACCAACTGGGTGTACCGAGGAGAGGCTGAGCGTATCTT
CATTGAGCTCAAGTTTACTGTACGTGACTGCAACAGCTTCCCTGGTGGCGCCAGCTC
CTGCAAGGAGACTTTCAACCTCTACTATGCCGAGTCGGACCTGGACTACGGCACCAA
CTTCCAGAAGCGCCTGTTACCAAGATTGACACCATTGCGCCCGATGAGATCACCGT
CAGCAGCGACTTCGAGGCACGCCACGTGAAGCTGAACGTGGAGGAGCGCTCCGTGG
GGCCGCTCACCCGCAAAGGCTTCTACCTGGCCTTCCAGGATATCGGTGCCTGTGTGG
CGTGCTCTCCGTCCGTGTCTACTACAAGAAGTGCCCCGAGCTGCTGCAGGGCCTGG
CCCACTTCCCTGAGACCATCGCCGGCTCTGATGCACCTTCCCTGGCCACTGTGGCCG
GCACCTGTGTGGACCATGCCGTGGTGCCACCGGGGGGTGAAGAGCCCCGTATGCAC
TGTGCAGTGGATGGCGAGTGGCTGGTGCCCATTTGGGCAGTGCCTGTGCCAGGCAGG
CTACGAGAAGGTGGAGGATGCCTGCCAGGCCTGCTCGCCTGGATTTTTTAAGTTTGA
GGCATCTGAGAGCCCCTGCTTGGAGTGCCCTGAGCACACGCTGCCATCCCCTGAGGG
TGCCACCTCCTGCGAGTGTGAGGAAGGCTTCTTCCGGGCACCTCAGGACCCAGCGTC
GATGCCTTGACACGACCCCCCTCCGCCCCACACTACCTCACAGCCGTGGGCATGGG
TGCCAAGGTGGAGCTGCGCTGGACGCCCCCTCAGGACAGCGGGGGCCGCGAGGACA
TTGTCTACAGCGTCACCTGCGAACAGTGCTGGCCCGAGTCTGGGGAATGCGGGCCGT
GTGAGGCCAGTGTGCGCTACTCGGAGCCTCCTCACGGACTGACCCGCACCAAGTGTG
ACAGTGAGCGACCTGGAGCCCCACATGAACTACACCTTCACCGTGGAGGCCCGCAA
TGGCGTCTCAGGCCTGGTAACCAGCCGCAGCTTCCGTACTGCCAGTGTGAGCATCAA
CCAGACAGAGCCCCCAAGGTGAGGCTGGAGGGCCGCAGCACCACTCGCTTAGCG
TCTCCTGGAGCATCCCCCGCCGCAGCAGAGCCGAGTGTGGAAGTACGAGGTCACT
TACCGCAAGAAGGGAGACTCCAACAGCTACAATGTGCGCCGCACCGAGGGTTTCTC
CGTGACCCTGGACGACCTGGCCCCAGACACCACCTACCTGGTCCAGGTGCAGGCAC
TGACGCAGGAGGGCCAGGGGGCCGGCAGGAGGTGCACGAATTCCAGACG

FIGURE 7

Construct: LLOss-PEST-EX2_hEphA2

Native LLO signal peptide + PEST fused to external domain of human EphA2

Not Codon optimized

No epitope tags (e.g., myc or FLAG used in this construct)

Predicted fusion protein shown

M K K I M L V F I T L I L V S L P I A Q Q T E A K D A S A F N K E N
S I S S M A P P A S P P A S P K T P I E K K H A D L E Q G K E V V L
L D F A A A G G E L G W L T H P Y G K G W D L M Q N I M N D M P I
Y M Y S V C N V M S G D Q D N W L R T N W V Y R G E A E R I F I E
L K F T V R D C N S F P G G A S S C K E T F N L Y Y A E S D L D Y G
T N F Q K R L F T K I D T I A P D E I T V S S D F E A R H V K L N V E
E R S V G P L T R K G F Y L A F Q D I G A C V A L L S V R V Y Y K K
C P E L L Q G L A H F P E T I A G S D A P S L A T V A G T C V D H A
V V P P G G E E P R M H C A V D G E W L V P I G Q C L C Q A G Y E
K V E D A C Q A C S P G F F K F E A S E S P C L E C P E H T L P S P
E G A T S C E C E E G F F R A P Q D P A S M P C T R P P S A P H Y L
T A V G M G A K V E L R W T P P Q D S G G R E D I V Y S V T C E Q
C W P E S G E C G P C E A S V R Y S E P P H G L T R T S V T V S D L
E P H M N Y T F T V E A R N G V S G L V T S R S F R T A S V S I N Q
T E P P K V R L E G R S T T S L S V S W S I P P P Q Q S R V W K Y E
V T Y R K K G D S N S Y N V R R T E G F S V T L D D L A P D T T Y
L V Q V Q A L T Q E G Q G A G S R V H E F Q T

FIGURE 8

NativeLLOss-PEST-FLAG-EX2_EphA2-myc-CodonOp

(Native L. monocytogenes LLO signal peptide + PEST-Codon optimized -FLAG-EX-2 EphA2-Myc)

Nucleotide Sequence (including *hly* promoter)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATTGCGTTTCATCTTTAGAAAGCGAATTCGCCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGAAAAAATAATGCTAGTTTTTATTACACTTATATTAGTTAGTCTA
CCAATTGCGCAACAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAAATTC
AATTTTCATCCATGGCACCACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGA
AAAGAAACACGCGGATGGATCCGATTATAAAGATGATGATGATAAACAAGGTAAAG
AAGTTGTTTTATTAGATTTTGCAGCAGCAGGTGGTGAATTAGGTTGGTTAACACATC
CATATGGTAAAGGTTGGGATTTAATGCAAATATTATGAATGATATGCCAATTTATA
TGTATAGTGTGTAATGTTATGAGTGGTGAATCAAGATAATTGGTTACGTACAAATT
GGGTTTATCGTGGTGAAGCAGAACGTATTTTTATTGAATTAAATTTACAGTTCGTG
ATTGTAATAGTTTTCCAGGTGGTGCAAGTAGTTGTAAAGAAACATTTAATTTATATT
ATGCAGAAAGTGATTTAGATTATGGTACAAATTTTCAAAAACGTTTATTTACAAAAA
TTGATACAATTGCACCAGATGAAATTACAGTTAGTAGTGATTTTGAAGCACGTCATG
TTAAATTAATGTTGAAGAACGTAGTGTTGGTCCATTAACACGTAAAGGTTTTTATT
TAGCATTTCAAGATATTGGTGCATGTGTTGCATTATTAAGTGTTTCGTGTTTATTATAA
AAAATGTCCAGAATTATTACAAGGTTTAGCACATTTCCAGAAACAATTGCAGGTAG
TGATGCACCAAGTTTAGCAACAGTTGCAGGTACATGTGTTGATCATGCAGTTGTTCC
ACCAGGTGGTGAAGAACCAGTATGCATTGTGCAGTTGATGGTGAATGTTAGTTCC
AATTGGTCAATGTTTATGTCAAGCAGGTTATGAAAAAGTTGAAGATGCATGTCAAGC
ATGTAGTCCAGGTTTTTTTAAATTTGAAGCAAGTGAAAGTCCATGTTTAGAATGTCC
AGAACATACATTACCAAGTCCAGAAGGTGCAACAAGTTGTGAATGTGAAGAAGGTT
TTTTTCGTGCACCACAAGATCCAGCAAGTATGCCATGTACACGTCCACCAAGTGCAC
CACATTATTTAACAGCAGTTGGTATGGGTGCAAAAGTTGAATTACGTTGGACACCAC
CACAAGATAGTGGTGGTCGTGAAGATATTGTTTATAGTGTTACATGTGAACAATGTT
GGCCAGAAAGTGGTGAATGTGGTCCATGTGAAGCAAGTGTTTCGTTATAGTGAACCA
CCACATGGTTTAACACGTACAAGTGTTACAGTTAGTGATTTAGAACCACATATGAAT
TATACATTTACAGTTGAAGCACGTAATGGTGTTAGTGGTTTAGTTACAAGTCGTAGT
TTTCGTACAGCAAGTGTTAGTATTAATCAAAACAGAACCAAAAAGTTCGTTTAGAA
GGTCGTAGTACAACAAGTTTAAAGTGTTAGTTGGAGTATTCACCACCACAACAAGT
CGTGTTTGGAATATGAAGTTACATATCGTAAAAAAGGTGATAGTAATAGTTATAAT
GTTTCGTCTACAGAAGGTTTTAGTGTTACATTAGATGATTTAGCACCAGATACAACA
TATTTAGTTCAAGTTCAAGCATTAACACAAGAAGGTCAAGGTGCAGGTAGTCGTGTT
CATGAATTTCAACAGAACAAAAATTAATTAGTGAAGAAGATTTATGAGAGCTC

FIGURE 9

NativeLLOss-PEST-FLAG-EX2_EphA2-myc-CodonOp
(Native L. monocytogenes LLO signal peptide + PEST-Codon optimized -FLAG-EX-2 EphA2-Myc)
Primary Amino Acid Sequence

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKEN
SISSMAPPASPPASPKTPIEKKHADGSDYKDDDD
KQGKEVVLLDFAAAGGELGWLTHPYGKGWDL
QNIMNDMPIYMYSVCNVMSGDQDNWLR TNWVY
RGEAERIFI ELKFTVRDCNSFPGGASSCKETFNL
YYAESDLDYGTNFQKRLFTKIDTIAPDEITVSSD
FEARHVKLNVEERSVGPLTRKGFYLA FQDIGACV
ALLSVRVYYKKCP ELLQGLAHFPETIAGSDAPSL
ATVAGTCVDH AVVPPGGEEPRMHCAVDGEWLVP
IGQCLCQAGYEKVEDACQACSPGFFKFEASESPC
LECPEHTLPSPEGATSCECEEGFFRAPQDPASMP
CTRPPSAPHYLTAVGMGAKVELRWTPPQDSGGR
EDIVYSVTCEQCWPESGECGPCEASVRYSEPPHG
LTRTSVTVS DLEPHMNYTFTVEARNGVSGLVTSR
SFRTASVSINQTEPPKVRLEGRSTTSLSVSW SIPP
PQQSRVWKYEVTYRKKGDSNSYNVRRTEGFSVT
LDDLAPD TTYLVQVQALTQEGQGAGSRVHEFQT
EQKLISEEDL

FIGURE 10

Codon Optimized LLOss-PEST-FLAG-EX2_EphA2-myc-CodonOp
(Codon Optimized *L. monocytogenes* LLO signal peptide + PEST-Codon optimized -FLAG-EX-2 EphA2-Myc)
Nucleotide Sequence (including *hly* promoter)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAGCGAATTTGCGCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGAAAAAAATTATGTTAGTTTTTTATTACATTAATTTTAGTTAGTTTAC
CAATTGCACAACAAACAGAAGCAAAAGATGCAAGTGCATTATAATAAGAAAATAGT
ATTAGTAGTATGGCACCACCAGCAAGTCCACCAGCAAGTCCAAAAACACCAATTGA
AAAAAACATGCAGATGGATCCGATTATAAAGATGATGATGATAACAAGGTAAAG
AAGTTGTTTTATTAGATTTTGCAGCAGCAGGTGGTGAATTAGGTTGGTTAACACATC
CATATGGTAAAGGTTGGGATTTAATGCAAAATATTATGAATGATATGCCAATTTATA
TGTATAGTGTGTTAATGTTATGAGTGGTGATCAAGATAATTGGTTACGTACAAATT
GGGTTTATCGTGGTGAAGCAGAACGTATTTTTATTGAATTAATAATTTACAGTTCGTG
ATTGTAATAGTTTTCCAGGTGGTGCAAGTAGTTGTAAAGAAACATTTAATTTATATT
ATGCAGAAAGTGATTTAGATTATGGTACAAATTTTCAAAAACGTTTATTTACAAAA
TTGATACAATTGCACCAGATGAAATTACAGTTAGTAGTGATTTTGAAGCACGTCATG
TTAAATTAATGTTGAAGAACGTAGTGTGGTCCATTAAACACGTAAAGGTTTTTATT
TAGCATTTCAGATATTGGTGCATGTTGTCATTATTAAGTGTTCGTGTTTATTATAA
AAAATGTCCAGAATTATTACAAGGTTTAGCACATTTTCCAGAAACAATTGCAGGTAG
TGATGCACCAAGTTTAGCAACAGTTGCAGGTACATGTGTTGATCATGCAGTTGTTCC
ACCAGGTGGTGAAGAACCACGTATGCATTGTGCAGTTGATGGTGAATGGTTAGTTCC
AATTGGTCAATGTTTATGTCAAGCAGGTTATGAAAAAGTTGAAGATGCATGTCAAGC
ATGTAGTCCAGGTTTTTTTAAATTTGAAGCAAGTGAAAGTCCATGTTTAGAATGTCC
AGAACATACATTACCAAGTCCAGAAGGTGCAACAAGTTGTGAATGTGAAGAAGGTT
TTTTTCGTGCACCACAAGATCCAGCAAGTATGCCATGTACACGTCCACCAAGTGCAC
CACATTATTTAACAGCAGTTGGTATGGGTGCAAAAGTTGAATTACGTTGGACACCAC
CACAAGATAGTGGTGGTTCGTGAAGATATTGTTTATAGTGTTACATGTGAACAATGTT
GGCCAGAAAGTGGTGAATGTGGTCCATGTGAAGCAAGTGTTTCGTTATAGTGAACCA
CCACATGGTTTAACACGTACAAGTGTTACAGTTAGTGATTTAGAACCACATATGAAT
TATACATTTACAGTTGAAGCACGTAATGGTGTAGTGGTTTAGTTACAAGTCGTAGT
TTTCGTACAGCAAGTGTTAGTATTAATCAAACAGAACCACCAAAAAGTTCGTTTAGAA
GGTCGTAGTACAACAAGTTTAAGTGTAGTTGGAGTATTCCACCACCACAACAAGT
CGTGTGTTGAAATATGAAGTTACATATCGTAAAAAAGGTGATAGTAATAGTTATAAT
GTTTCGTCTACAGAAGGTTTTAGTGTACATTAGATGATTTAGCACCAGATACAACA
TATTTAGTTCAAGTTCAAGCATTAACACAAGAAGGTCAAGGTGCAGGTAGTCGTGT
CATGAATTTCAAACAGAACAAAAATTAATTAGTGAAGAAGATTTATGAGAGCTC

FIGURE 11

Codon Optimized LLOss-PEST-FLAG-EX2_EphA2-myc-CodonOp
(Codon Optimized *L. monocytogenes* LLO signal peptide + PEST-Codon optimized -FLAG-EX-2 EphA2-Myc)
Primary Amino Acid Sequence

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKEN
SISSMAPPASPPASPKTPIEKKHADGSDYKDDDD
KQGKEVVLLDFAAAGGELGWLTHPYGKGWDL
QNIMNDMPIYMYSVCNVMSGDQDNWLRTNWVY
RGEAERIFIELKFTVRDCNSFPGGASSCKETFNL
YYAESDLDYGTNFKRLFTKIDTIAPDEITVSSD
FEARHVKLNVEERSVGPLTRKGFYLAHQDIGACV
ALLSVRVYYKKCPPELLQGLAHFPETIAGSDAPSL
ATVAGTCVDHAVVPPGGEEPRLMHCAVDGEWLVP
IGQCCLCQAGYEKVEDACQACSPGFFKFEASESPC
LECPEHTLPSPEGATSCECEEGFFRAPQDPASMP
CTRPPSAPHYLTAVGMGAKVELRWTPPQDSGGR
EDIVYSVTCEQCWPESGECGPCEASVRYSEPPHG
LTRTSVTVSDLEPHMNYTFTVEARNGVSGLVTSR
SFRTASVSINQTEPPKVRLEGRSTTSLSVSWSIPP
PQQSRVWKYEVTYRKKGDSNSYNVRRTEGFSVT
LDDLAPDTTYLVQVQALTQEGQGAGSRVHEFQT
EQKLISEEDL

FIGURE 12

PhoD-FLAG-EX2_EphA2-myc-CodonOp

(Codon optimized B. subtilis phoD Tat signal peptide-FLAG-EX-2 EphA2-Myc)

Nucleotide Sequence (including *hly* promoter)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAGCGAATTTTCGCCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGGCATAACGACAGTCGTTTTGATGAATGGGTACAGAACTGAAAGA
GGAAAGCTTTCAAAACAATACGTTTGACCGCCGCAAATTTATTCAAGGAGCGGGGA
AGATTGCAGGACTTTCTCTTGGATTAACGATTGCCAGTCGGTTGGGGCCTTTGGAT
CCGATTATAAAGATGATGATGATAACAAGGTAAAGAAGTTGTTTTATTAGATTTTG
CAGCAGCAGGTGGTGAATTAGGTTGGTTAACACATCCATATGGTAAAGGTTGGGATT
TAATGCAAAATATTATGAATGATATGCCAATTTATATGTATAGTGTGTGAATGTTAT
GAGTGGTGATCAAGATAATTGGTTACGTACAAATTGGGTTTATCGTGGTGAAGCAGA
ACGTATTTTTATTGAATTAATAATTACAGTTCGTGATTGTAATAGTTTTCCAGGTGGT
GCAAGTAGTTGTAAAGAAACATTTAATTTATATTATGCAGAAAGTGATTTAGATTAT
GGTACAAATTTTCAAAAACGTTTATTTACAAAAATTGATACAATTGCACCAGATGAA
ATTACAGTTAGTAGTGATTTTGAAGCACGTCATGTTAAATTAAATGTTGAAGAACGT
AGTGTGGTCCATTAACACGTAAAGGTTTTTATTTAGCATTTCAGATATTGGTGCAT
GTGTTGCATTATTAAGTGTTTCGTGTTTATTATAAAAAATGTCCAGAATTATTACAAG
GTTTAGCACATTTTCCAGAAACAATTGCAGGTAGTGATGCACCAAGTTTAGCAACAG
TTGCAGGTACATGTGTTGATCATGCAGTTGTTCCACCAGGTGGTGAAGAACCACGTA
TGCATTGTGCAGTTGATGGTGAATGGTTAGTTCCAATTGGTCAATGTTTATGTCAAG
CAGGTTATGAAAAAGTTGAAGATGCATGTCAAGCATGTAGTCCAGGTTTTTTTAAAT
TTGAAGCAAGTGAAAGTCCATGTTTAGAATGTCCAGAACATACATTACCAAGTCCAG
AAGGTGCAACAAGTTGTGAATGTGAAGAAGGTTTTTTTCGTGCACCACAAGATCCAG
CAAGTATGCCATGTACACGTCCACCAAGTGCACCACATTATTTAACAGCAGTTGGTA
TGGGTGCAAAAGTTGAATTACGTTGGACACCACCACAAGATAGTGGTGGTCGTGAA
GATATTGTTTATAGTGTTACATGTGAACAATGTTGGCCAGAAAGTGGTGAATGTGGT
CCATGTGAAGCAAGTGTTTCGTTATAGTGAACCACCACATGGTTTAACACGTACAAGT
GTTACAGTTAGTGATTTAGAACCACATATGAATTATACATTTACAGTTGAAGCACGT
AATGGTGTTAGTGTTTTAGTTACAAGTCGTAGTTTTTCGTACAGCAAGTGTTAGTATT
AATCAAAACAGAACCAACCAAAAGTTTCGTTTAGAAGGTCGTAGTACAACAAGTTTAAAG
TGTTAGTTGGAGTATTCCACCACCACAACAAAGTCGTGTTTGGAAATATGAAGTTAC
ATATCGTAAAAAAGGTGATAGTAATAGTTATAATGTTTCGTTCGTACAGAAGGTTTTAG
TGTTACATTAGATGATTTAGCACCAGATACAACATATTTAGTTCAAGTTCAAGCATT
AACACAAGAAGGTCAAGGTGCAGGTAGTCGTGTTTCATGAATTTCAAACAGAACAAA
AATTAATTAGTGAAGAAGATTTATGAGAGCTC

FIGURE 13

PhoD-FLAG-EX2_EphA2-myc-CodonOp

(Codon optimized B. subtilis phoD Tat signal peptide-FLAG-EX-2 EphA2-Myc)

Amino acid sequence

M A Y D S R F D E W V Q K L K E E S F Q N N T F D R R K F I Q G A
G K I A G L S L G L T I A Q S V G A F G S D Y K D D D D K Q G K E
V V L L D F A A A G G E L G W L T H P Y G K G W D L M Q N I M N
D M P I Y M Y S V C N V M S G D Q D N W L R T N W V Y R G E A E
R I F I E L K F T V R D C N S F P G G A S S C K E T F N L Y Y A E S
D L D Y G T N F Q K R L F T K I D T I A P D E I T V S S D F E A R H
V K L N V E E R S V G P L T R K G F Y L A F Q D I G A C V A L L S V
R V Y Y K K C P E L L Q G L A H F P E T I A G S D A P S L A T V A G
T C V D H A V V P P G G E E P R M H C A V D G E W L V P I G Q C L
C Q A G Y E K V E D A C Q A C S P G F F K F E A S E S P C L E C P E
H T L P S P E G A T S C E C E E G F F R A P Q D P A S M P C T R P P
S A P H Y L T A V G M G A K V E L R W T P P Q D S G G R E D I V Y
S V T C E Q C W P E S G E C G P C E A S V R Y S E P P H G L T R T S
V T V S D L E P H M N Y T F T V E A R N G V S G L V T S R S F R T A
S V S I N Q T E P P K V R L E G R S T T S L S V S W S I P P P Q Q S R
V W K Y E V T Y R K K G D S N S Y N V R R T E G F S V T L D D L A
P D T T Y L V Q V Q A L T Q E G Q G A G S R V H E F Q T E Q K L I S
E E D L

FIGURE 14

EphA2 CO domain
Native nucleotide sequence

CACCGCAGGAGGAAGAACCAGCGTGCCCGCCAGTCCCCGGAGGACGTTTACTTCTC
CAAGTCAGAACAACTGAAGCCCCTGAAGACATACGTGGACCCCCACACATATGAGG
ACCCCAACCAGGCTGTGTTGAAGTTCCTACCGAGATCCATCCATCCTGTGTCACTC
GGCAGAAGGTGATCGGAGCAGGAGAGTTTGGGGAGGTGTACAAGGGGCATGCTGAA
GACATCCTCGGGGAAGAAGGAGGTGCCGGTGGCCATCAAGACGCTGAAAGCCGGCT
ACACAGAGAAGCAGCGAGTGGACTTCCTCGGCGAGGCCGGCATCATGGGCCAGTTC
AGCCACCACAACATCATCCGCTAGAGGGCGTCATCTCCAAATACAAGCCCATGAT
GATCATCACTGAGTACATGGAGAATGGGGCCCTGGACAAGTTCCTTCGGGAGAAGG
ATGGCGAGTTCAGCGTGCTGCAGCTGGTGGGCATGCTGCGGGGCATCGCAGCTGGC
ATGAAGTACCTGGCCAACATGAACTATGTGCACCGTGACCTGGCTGCCCGCAACATC
CTCGTCAACAGCAACCTGGTCTGCAAGGTGTCTGACTTTGGCCTGTCCCGCGTGCTG
GAGGACGACCCCGAGGCCACCTACACCACAGTGGCGGCAAGATCCCCATCCGCTG
GACCGCCCCGGAGGCCATTTCTACCGGAAGTTCACCTCTGCCAGCGACGTGTGGAG
CTTTGGCATTGTCTATGTGGGAGGTGATGACCTATGGCGAGCGGCCCTACTGGGAGTT
GTCCAACCACGAGGTGATGAAAGCCATCAATGATGGCTTCCGGCTCCCCACACCCAT
GGACTGCCCCCTCCGCCATCTACCAGCTCATGATGCAGTGCTGGCAGCAGGAGCGTGC
CCGCCGCCCAAGTTCGCTGACATCGTCAGCATCCTGGACAAGCTCATTCGTGCCCC
TGACTCCCTCAAGACCCTGGCTGACTTTGACCCCCGCGTGTCTATCCGGCTCCCCAG
CACGAGCGGCTCGGAGGGGGTGCCCTTCCGCACGGTGTCCGAGTGGCTGGAGTCCA
TCAAGATGCAGCAGTATACGGAGCACTTCATGGCGGCCGGCTACACTGCCATCGAG
AAGGTGGTGCAGATGACCAACGACGACATCAAGAGGATTGGGGTGCGGCTGCCCGG
CCACCAGAAGCGCATCGCCTACAGCCTGCTGGGACTCAAGGACCAGGTGAACACTG
TGGGGATCCCCATC

FIGURE 15

EphA2 CO domain

Nucleotide sequence for optimal codon usage in *Listeria*

CACAGACGTAGAAAAAATCAACGTGCTCGACAATCCCCAGAAGATGTGTATTTTTTCG
AAAAGTGAACAATTAACCATTAAAACTTATGTTGATCCGCATACGTACGAAGA
CCCAAATCAAGCAGTATTAATAATTTACAACAGAAATACACCCAAGTTGTGTTACAA
GACAAAAAGTTATTGGAGCAGGTGAATTCGGAGAGGTATATAAAGGTATGTTAAAA
ACATCATCAGGTAAAAAGAAGTTCCGGTTGCAATTAACCTTAAGGCAGGATA
TACAGAAAAACAGCGAGTTGATTTTTTAGGTGAAGCAGGAATTATGGGTCAATTTAG
CCATCATAATATTATTCGTTTGGAAAGGAGTAATAAGTAAATATAAACCAATGATGAT
TATTACAGAATACATGGAAAACGGTGCTTTAGATAAATTTTTACGTGAAAAGGATGG
TGAATTTAGTGTTTTACAATTGGTTGGTATGTTAAGAGGAATTGCTGCAGGTATGAA
ATATTTAGCTAATATGAATTATGTTCCCGTGATTTGGCAGCAAGAAATATCCTAGT
CAATTCCAATTTAGTATGTAAAGTTAGTGATTTTGGTTTAAGCAGAGTATTAGAAGA
CGATCCAGAGGCAACCTATACAACATCGGGAGGTAAAATTCCTATTCGTTGGACAG
CACCAGAAGCTATCAGTTACCGTAAATTTACAAGTGCATCAGACGTGTGGAGTTTTG
GGATTGTAATGTGGGAAGTTATGACATATGGAGAAAGACCATATTGGGAATTAAGT
AATCATGAAGTTATGAAAGCAATTAACGATGGATTTAGATTACCAACTCCGATGGAT
TGTCATCTGCCATTTATCAACTAATGATGCAATGTTGGCAACAAGAAAGAGCACGA
CGTCCAAAATTTGCAGATATTGTTAGTATTTTAGACAAATTAATTCGTGCACCAGAT
AGTTTAAAACTTTAGCAGACTTTGATCCTCGTGTTAGTATTCGATTACCAAGTACGT
CAGGTCCGAAGGAGTTCCATTTTCGCACAGTCTCCGAATGGTTGGAATCAATTAATA
TGCAACAATACACCGAACACTTTATGGCAGCAGGTTACACAGCAATCGAAAAAGTT
GTTCAAATGACAAATGATGATATTAAACGTATTGGAGTTAGATTACCAGGCCACCAG
AAACGTATTGCATATTCTTTATTAGGTTTAAAAGATCAAGTTAATACCGTGGAATT
CCAATT

FIGURE 16

EphA2 CO domain
Primary Amino Acid Sequence

V H E F Q T L S P E G S G N L A V I G G V A V G V V L L L V L A G V
G F F I H R R R K N Q R A R Q S P E D V Y F S K S E Q L K P L K T Y
V D P H T Y E D P N Q A V L K F T T E I H P S C V T R Q K V I G A G
E F G E V Y K G M L K T S S G K K E V P V A I K T L K A G Y T E K
Q R V D F L G E A G I M G Q F S H H N I I R L E G V I S K Y K P M M
I I T E Y M E N G A L D K F L R E K D G E F S V L Q L V G M L R G I
A A G M K Y L A N M N Y V H R D L A A R N I L V N S N L V C K V S
D F G L S R V L E D D P E A T Y T T S G G K I P I R W T A P E A I S
Y R K F T S A S D V W S F G I V M W E V M T Y G E R P Y W E L S N
H E V M K A I N D G F R L P T P M D C P S A I Y Q L M M Q C W Q Q
E R A R R P K F A D I V S I L D K L I R A P D S L K T L A D F D P R
V S I R L P S T S G S E G V P F R T V S E W L E S I K M Q Q Y T E H
F M A A G Y T A I E K V V Q M T N D D I K R I G V R L P G H Q K R I
A Y S L L G L K D Q V N T V G I P I

FIGURE 17

Construct: LLOss-PEST-CO-huEphA2

Native LLO signal peptide + PEST fused to cytoplasmic domain of human EphA2

Not Codon optimized

No epitope tags (e.g., myc or FLAG used in this construct)

Fusion protein coding sequence shown

ATGAAAAAATAATGCTAGTTTTTTATTACACTTATATTAGTTAGTCTACCAATTGCGC
AACAAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAATTCAATTTTCATCC
ATGGCACCACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGAAAAGAAACA
CGCGGATCTCGAGCACCGCAGGAGGAAGAACCAGCGTGCCCGCCAGTCCCCGGAGG
ACGTTTACTTCTCCAAGTCAGAACAACCTGAAGCCCCCTGAAGACATACGTGGACCCCC
ACACATATGAGGACCCCAACCAGGCTGTGTTGAAGTTCACTACCGAGATCCATCCAT
CCTGTGTCACTCGGCAGAAGGTGATCGGAGCAGGAGAGTTTGGGGAGGTGTACAAG
GGCATGCTGAAGACATCCTCGGGGAAGAAGGAGGTGCCGGTGGCCATCAAGACGCT
GAAAGCCGGCTACACAGAGAAGCAGCGAGTGGACTTCCTCGGCGAGGCCGGCATCA
TGGGCCAGTTCAGCCACCACAACATCATCCGCCTAGAGGGCGTCATCTCCAAATACA
AGCCCATGATGATCATCACTGAGTACATGGAGAATGGGGCCCTGGACAAGTTCCTTC
GGGAGAAGGATGGCGAGTTCAGCGTGCTGCAGCTGGTGGGCATGCTGCGGGGCATC
GCAGCTGGCATGAAGTACCTGGCCAACATGAACTATGTGCACCGTGACCTGGCTGC
CCGCAACATCCTCGTCAACAGCAACCTGGTCTGCAAGGTGTCTGACTTTGGCCTGTC
CCGCGTGCTGGAGGACGACCCCGAGGCCACCTACACCACCAGTGGCGGCAAGATCC
CCATCCGCTGGACCGCCCCGGAGGCCATTTCTACCGGAAGTTCACCTCTGCCAGCG
ACGTGTGGAGCTTTGGCATTTGTCATGTGGGAGGTGATGACCTATGGCGAGCGGCCCT
ACTGGGAGTTGTCCAACCACGAGGTGATGAAAGCCATCAATGATGGCTTCCGGCTCC
CCACACCCATGGACTGCCCCCTCCGCCATCTACCAGCTCATGATGCAGTGCTGGCAGC
AGGAGCGT'GCCCCGCCGCCCAAGTTCGCTGACATCGTCAGCATCCTGGACAAGCTC
ATTCGTGCCCCTGACTCCCTCAAGACCCTGGCTGACTTTGACCCCCGCGTGTCTATCC
GGCTCCCCAGCACGAGCGGCTCGGAGGGGGTGGCCCTTCCGCACGGTGTCCGAGTGG
CTGGAGTCCATCAAGATGCAGCAGTATACGGAGCACTTCATGGCGGCCGGCTACAC
TGCCATCGAGAAGGTGGTGCAGATGACCAACGACGACATCAAGAGGATTGGGGTGC
GGCTGCCCCGGCCACCAGAAGCGCATCGCCTACAGCCTGCTGGGACTCAAGGACCAG
GTGAACACTGTGGGGATCCCCATC

FIGURE 18

Construct: LLOss-PEST-CO-huEphA2

Native LLO signal peptide + PEST fused to cytoplasmic domain of human EphA2

Not Codon optimized

No epitope tags (e.g., myc or FLAG used in this construct)

Predicted fusion protein shown

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKEN
SISSMAPPASPPASPKTPIEKKHADLEHRRRK NQ
RARQSPEDVYFSKSEQLKPLKTYVDPHTYEDPNQ
AVLKFTTEIHPS CVTRQKVIGAGEFGGEVYKGMLK
TSSGKKEVPVAIKTLKAGYTEKQRVDFLGEAGIM
GQFSHHNIIRLEGVISKYKPMMIITEY MENGALD
KFLREKDG EFSVLQLVGMLRGIAAGMKYLANMN
YVHRDLAARNILVNSNLVCKVSDFGLSRVLEDDP
EATYTTSGGKIPIRWTAPEAISYRKFTSASDVWS
FGIVMWEVMTYGERPYWELSNHEVMKAINDGFR
LPTPMDCPSAIYQLMMQCWQQERARRPKFADIV
SILDKLIRAPDSLKTLADFDPRVSIRLPSTSGSEG
VPFRTVSEWLESIKMQQYTEHFMAAGYTAIEKV
VQMTNDDIKRIGVRLPGHQKRIAYSLLGLKDQVN
TVGIP I

FIGURE 19

NativeLLOss-PEST-FLAG-CO_EphA2-myc-CodonOp
 (Native L. monocytogenes LLO signal peptide + PEST-Codon optimized -FLAG-CO_EphA2-
 Myc)
 Nucleotide Sequence (including *hly* promoter)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
 ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
 CATATAATATTGCGTTTCATCTTTAGAAGCGAATTTGCGCAATATTATAATTATCAAA
 AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTAAAAAATGTAGAAGGAGA
 GTGAAACCCATGAAAAAATAATGCTAGTTTTTATTACACTTATATTAGTTAGTCTA
 CCAATTGCGCAACAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAAATTC
 AATTTTCATCCATGGCACCACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGA
 AAAGAAACACGCGGATGGATCCGATTATAAAGATGATGATGATAAACACAGACGTA
 GAAAAAATCAACGTGCTCGACAATCCCCAGAAGATGTGTATTTTTTCGAAAAAGTGAA
 CAATTAAGAACCTTAAAAACTTATGTTGATCCGCATACGTACGAAGACCCAAATCAA
 GCAGTATTAAAAATTTACAACAGAAATACACCCAAGTTGTGTTACAAGACAAAAAGT
 TATTGGAGCAGGTGAATTCGGAGAGGTATATAAAGGTATGTTAAAAACATCATCAG
 GTAAAAAAGAAGTTCCGGTTGCAATTA AAAACCTTAAAGGCAGGATATACAGAAAAA
 CAGCGAGTTGATTTTTTAGGTGAAGCAGGAATTATGGGTCAATTTAGCCATCATAAT
 ATTATTCGTTTGGAAGGAGTAATAAGTAAATATAAACCAATGATGATTATTACAGAA
 TACATGGAAAAACGGTGCTTTAGATAAAATTTTACGTGAAAAGGATGGTGAATTTAGT
 GTTTTACAATTGGTTGGTATGTTAAGAGGAATTGCTGCAGGTATGAAATATTTAGCT
 AATATGAATTATGTTACCGTGATTTGGCAGCAAGAAATATCCTAGTCAATTCCAAT
 TTAGTATGTAAAGTTAGTGATTTTGGTTTAAAGCAGAGTATTAGAAGACGATCCAGAG
 GCAACCTATACAACATCGGGAGGTAAAAATTCCTATTCGTTGGACAGCACCAGAAGC
 TATCAGTTACCGTAAATTTACAAGTGCATCAGACGTGTGGAGTTTTGGGATTGTAAT
 GTGGGAAGTTATGACATATGGAGAAAGACCATATTGGGAATTAAGTAATCATGAAG
 TTATGAAAGCAATTAACGATGGATTTAGATTACCAACTCCGATGGATTGTCCATCTG
 CCATTTATCAACTAATGATGCAATGTTGGCAACAAGAAAGAGCACGACGTCCAAAA
 TTTGCAGATATTGTTAGTATTTTAGACAAATTAATTCGTGCACCAGATAGTTTAAAA
 ACTTTAGCAGACTTTGATCCTCGTGTTAGTATTCGATTACCAAGTACGTACAGTTCCG
 AAGGAGTTCCATTTTCGCACAGTCTCCGAATGGTTGGAATCAATTA AAAATGCAACAAT
 ACACCGAACACTTTATGGCAGCAGGTTACACAGCAATCGAAAAAGTTGTTCAAATG
 ACAAATGATGATATTAAACGTATTGGAGTTAGATTACCAGGCCACCAGAAACGTATT
 GCATATTCTTTATTAGGTTTAAAAGATCAAGTTAATACCGTGGGAATTCCAATTGAA
 CAAAAATTAATTTCCGAAGAAGACTTATAAGAGCTC

FIGURE 20

NativeLLOss-PEST-FLAG-CO_EphA2-myc-CodonOp

(Native *L. monocytogenes* LLO signal peptide + PEST-Codon optimized -FLAG-CO_EphA2-Myc)

Primary Amino Acid Sequence

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKEN
SISSMAPPASPPASPKTPIEKKHADGSDYKDDDD
KHRRRKNQRARQSPEDVYFSKSEQLKPLKTYVD
PHTYEDPNQAVLKFTTEIHPSCVTRQKVIGAGEF
GEVYKGM LKTSSGKKEVPVAIKTLKAGYTEKQR
VDLFLGEAGIMGQFSHHNIIRLEGVISKYKPMMIIT
EYMEENGALDKFLREKDGFEFSVLQLVGMLRGIAA
GMKYL ANMNYVHRDLAARNILVNSNLVCKVSDF
GLSRVLEDDPEATYTTSGGKIPIRWTAPEAISYR
KFTSASDVWSFGIVMWEVMTYGERPYWELSNHE
VMKAINDGFRLPTPMDCPSAIYQLMMQCWQQR
ARRPKFADIVSILDKLIRAPDSLKTLDLDFDPRVSI
RLPSTSGSEGVPFRTVSEWLESIKMQQYTEHFMA
AGYTAIEKVVQMTNDDIKRIGVRLPGHQKRIAYS
LLGLKDQVNTVGIPIEQKLISEEDL

FIGURE 21

Codon Optimized LLOss-PEST-FLAG-CO_EphA2-myc-CodonOp
 (Codon Optimized *L. monocytogenes* LLO signal peptide + PEST-Codon optimized -FLAG-
 CO_EphA2-Myc)
 Nucleotide Sequence (including *hly* promoter)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
 ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
 CATATAATATTGCGTTTCATCTTTAGAAAGCGAATTTGCGCAATATTATAATTATCAAA
 AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
 GTGAAACCCATGAAAAAAATTATGTTAGTTTTTATTACATTAATTTTAGTTAGTTTAC
 CAATTGCACAACAAACAGAAGCAAAAGATGCAAGTGCATTTAATAAAGAAAAATAGT
 ATTAGTAGTATGGCACCACCAGCAAGTCCACCAGCAAGTCCAAAAACACCAATTGA
 AAAAAACATGCAGATGGATCCGATTATAAAGACGATGATGATAAACACAGACGTA
 GAAAAAATCAACGTGCTCGACAATCCCCAGAAGATGTGTATTTTCGAAAAAGTGAA
 CAATTAAAACCATTA AAAA ACTTATGTTGATCCGCATACGTACGAAGACCCAAATCAA
 GCAGTATTA AAAATTTACAACAGAAATACACCCAAGTTGTGTTACAAGACAAAAAGT
 TATTGGAGCAGGTGAATTCGGAGAGGTAATAAAGGTATGTTAAAAACATCATCAG
 GTAAAAAAGAAGTTCCGGTTGCAATTA AACCTTAAAGGCAGGATATACAGAAAAA
 CAGCGAGTTGATTTTTTAGGTGAAGCAGGAATTATGGGTCAATTTAGCCATCATAAT
 ATTATTCGTTTGAAGGAGTAATAAGTAAATATAAACCAATGATGATTATTACAGAA
 TACATGGAAAACGGTGCTTTAGATAAATTTTACGTGAAAAGGATGGTGAATTTAGT
 GTTTTACAATTGGTTGGTATGTTAAGAGGAATTGCTGCAGGTATGAAATATTTAGCT
 AATATGAATTATGTTACCGTGATTTGGCAGCAAGAAATATCCTAGTCAATTCCAAT
 TTAGTATGTAAAGTTAGTGATTTTGGTTAAGCAGAGTATTAGAAGACGATCCAGAG
 GCAACCTATACAACATCGGGAGGTAAAATTCCCTATTCGTTGGACAGCACCAGAAGC
 TATCAGTTACCGTAAATTTACAAGTGCATCAGACGTGTGGAGTTTTGGGATTGTAAT
 GTGGGAAGTTATGACATATGGAGAAAGACCATATTGGGAATTAAGTAATCATGAAG
 TTATGAAAGCAATTAACGATGGATTTAGATTACCAACTCCGATGGATTGTCCATCTG
 CCATTTATCAACTAATGATGCAATGTTGGCAACAAGAAAGAGCACGACGTCCAAAA
 TTTGCAGATATTGTTAGTATTTTAGACAAATTAATTCGTGCACCAGATAGTTTAAAA
 ACTTTAGCAGACTTTGATCCTCGTGTTAGTATTCGATTACCAAGTACGTCAGGTTCCG
 AAGGAGTTCCATTTTCGCACAGTCTCCGAATGGTTGGAATCAATTA AAAATGCAACAAT
 ACACCGAACACTTTATGGCAGCAGGTTACACAGCAATCGAAAAAGTTGTTCAAATG
 ACAAATGATGATATTA AACGTATTGGAGTTAGATTACCAGGCCACCAGAAACGTATT
 GCATATTCTTTATTAGGTTTAAAAGATCAAGTTAATACCGTGGGAATTC CAATTGAA
 CAAAAATTAATTTCCGAAGAAGACTTATAAGAGCTC

FIGURE 22

Codon Optimized LLOss-PEST-FLAG-CO_EphA2-myc-CodonOp
(Codon Optimized L. monocytogenes LLO signal peptide + PEST-Codon optimized -FLAG-
CO_EphA2-Myc)
Primary Amino Acid Sequence

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKEN
SISSMAPPASPPASPKTPIEKKHADGSDYKDDDD
KHRRRKNNQRARQSPEDVYFSKSEQLKPLKTYVD
PHTYEDPNQAVLKFTTEIHPSCVTRQKVIGAGEF
GEVYKGMLKTSSGKKEVPVAIKTLKAGYTEKQR
VDNFLGEAGIMGQFSHHNIIRLEGVISKYKPMMIIT
EYMENGALDKFLREKDGESVLQLVGMLRGIAA
GMKYLANMNYVHRDLAARNILVNSNLVCKVSDF
GLSRVLEDDPEATYTTSGGKIPIRWTAPEAISYR
KFTSASDVWSFGIVMWEVMTYGERPYWELSNHE
VMKAINDGFRLPTPMDCPSAIYQLMMQCWQQR
ARRPKFADIVSILDKLIRAPDSLKTLADFDPRVSI
RLPSTSGSEGVPFRTVSEWLESIKMQQYTEHFMA
AGYTAIEKVVQMTNDDIKRIGVRLPGHQKRIAYS
LLGLKDQVNTVGIPIEQKLISEEDL

FIGURE 23

PhoD-FLAG-CO_EphA2-myc-CodonOp

(Codon optimized B. subtilis phoD Tat signal peptide-FLAG-CO_EphA2-Myc)

Nucleotide Sequence (including *hly* promoter)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAGCGAATTTGCGCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGGCATAACGACAGTCGTTTTGATGAATGGGTACAGAAACTGAAAGA
GGAAAGCTTTCAAAACAATACGTTTGACCGCCGCAAATTTATTCAAGGAGCGGGGA
AGATTGCAGGACTTTCTCTTGGATTAACGATTGCCAGTCGGTTGGGGCCTTTGGAT
CCGATTATAAAGATGATGATGATAAACACAGACGTAGAAAAAATCAACGTGCTCGA
CAATCCCCAGAAGATGTGTATTTTTCGAAAAAGTGAACAATTAACCATTAAAAACT
TATGTTGATCCGCATACGTACGAAGACCCAAATCAAGCAGTATTAATTTACAACA
GAAATACACCCAAGTTGTGTTACAAGACAAAAAGTTATTGGAGCAGGTGAATTCGG
AGAGGTATATAAAGGTATGTTAAAAACATCATCAGGTAAAAAAGAAGTTCGGTTG
CAATTAACCTTAAAGGCAGGATATACAGAAAAACAGCGAGTTGATTTTTAGGT
GAAGCAGGAATTATGGGTCAATTTAGCCATCATAATATTATTCGTTTGAAGGAGTA
ATAAGTAAATATAAACCAATGATGATTATTACAGAATACATGGAAAACGGTGCTTT
AGATAAATTTTACGTGAAAAGGATGGTGAATTTAGTGTTTACAATTGGTTGGTAT
GTTAAGAGGAATTGCTGCAGGTATGAAATATTTAGCTAATATGAATTATGTTACCG
TGATTTGGCAGCAAGAAATATCCTAGTCAATTTCCAATTTAGTATGTAAGTTAGTGA
TTTTGGTTTAAAGCAGAGTATTAGAAGCGATCCAGAGGCAACCTATACAACATCGG
GAGGTAAAATTCCTATTCGTTGGACAGCACCAGAAGCTATCAGTTACCGTAAATTTA
CAAGTGCATCAGACGTGTGGAGTTTTGGGATTGTAATGTGGGAAGTTATGACATATG
GAGAAAGACCATATTGGGAATTAAGTAATCATGAAGTTATGAAAGCAATTAACGAT
GGATTTAGATTACCAACTCCGATGGATTGTCCATCTGCCATTTATCAACTAATGATG
CAATGTTGGCAACAAGAAAGAGCACGACGTCCAAAATTTGCAGATATTGTTAGTATT
TTAGACAAATTAATTCGTGCACCAGATAGTTTAAAAACTTTAGCAGACTTTGATCCT
CGTGTTAGTATTCGATTACCAAGTACGTCAGGTTCCGAAGGAGTTCCATTTGCACA
GTCTCCGAATGGTTGGAATCAATTAATAATGCAACAATACACCGAACACTTTATGGCA
GCAGGTTACACAGCAATCGAAAAAGTTGTTCAAATGACAAATGATGATATTAAACG
TATTGGAGTTAGATTACCAGGCCACCAGAAACGTATTGCATATTCTTTATTAGGTTT
AAAAGATCAAGTTAATACCGTGGGAATTCCAATTGAACAAAAATTAATTTCCGAAG
AAGACTTATAAGAGCTC

FIGURE 24

PhoD-FLAG-CO_EphA2-myc-CodonOp

(Codon optimized B. subtilis phoD Tat signal peptide-FLAG-CO_EphA2-Myc)

Amino acid sequence

M A Y D S R F D E W V Q K L K E E S F Q N N T F D R R K F I Q G A
G K I A G L S L G L T I A Q S V G A F G S D Y K D D D D K H R R R
K N Q R A R Q S P E D V Y F S K S E Q L K P L K T Y V D P H T Y E
D P N Q A V L K F T T E I H P S C V T R Q K V I G A G E F G E V Y K
G M L K T S S G K K E V P V A I K T L K A G Y T E K Q R V D F L G
E A G I M G Q F S H H N I I R L E G V I S K Y K P M M I I T E Y M E
N G A L D K F L R E K D G E F S V L Q L V G M L R G I A A G M K Y
L A N M N Y V H R D L A A R N I L V N S N L V C K V S D F G L S R
V L E D D P E A T Y T T S G G K I P I R W T A P E A I S Y R K F T S
A S D V W S F G I V M W E V M T Y G E R P Y W E L S N H E V M K
A I N D G F R L P T P M D C P S A I Y Q L M M Q C W Q Q E R A R R
P K F A D I V S I L D K L I R A P D S L K T L A D F D P R V S I R L P
S T S G S E G V P F R T V S E W L E S I K M Q Q Y T E H F M A A G Y
T A I E K V V Q M T N D D I K R I G V R L P G H Q K R I A Y S L L G
L K D Q V N T V G I P I E Q K L I S E E D L

FIGURE 25

Codon Optimized LLOss-PEST-NYESO1-CodonOp
(Codon Optimized *L. monocytogenes* LLO signal peptide + PEST-Codon optimized -NYESO1)
Nucleotide Sequence (including *hly* promoter)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAAGCGAATTTGCGCAATATTATAATTATCAAA
AGAGAGGGGTGCGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGAAAAAAATTATGTTAGTTTTTATTACATTAATTTTAGTTAGTTTAC
CAATTGCACAACAAACAGAAGCAAAAGATGCAAGTGCATTTAATAAAGAAAAATAGT
ATTAGTAGTATGGCACCACCAGCAAGTCCACCAGCAAGTCCAAAAACACCAATTGA
AAAAAAACATGCAGATGGATCCCAAGCAGAAGGTCGCGGAACAGGAGGAAGTACA
GGAGATGCAGACGGACCAGGAGGACCAGGAATACCAGACGGACCAGGAGGAAATG
CAGGAGGCCCAGGCGAAGCAGGCGCAACAGGAGGAAGAGGACCAAGAGGAGCAG
GAGCAGCACGAGCATCAGGACCAGGAGGCGGAGCACCAAGAGGACCACATGGCGG
AGCGGCAAGCGGATTAAATGGATGTTGTAGATGTGGAGCACGCGGACCAGAATCAA
GACTTTTAGAATTTTATTTAGCCATGCCATTTGCAACCCCAATGGAAGCAGAATTAG
CACGAAGATCATTAGCACAAGATGCCCCACCATTACCAGTACCAGGAGTTTTATTAA
AAGAGTTTACAGTATCAGGCAATATTTTAAACAATACGTTTAACAGCAGCAGACCATC
GTCAATTACAACATCTATCAGTTCATGTTTACAACAATTATCCTTATTAATGTGGAT
TACACAATGTTTTTTACCAGTTTTTTTAGCACAACCACCATCAGGACAAAGAAGATA
AGAGCTC

FIGURE 26

Codon Optimized LLOss-PEST-NYESO1-CodonOp
(Codon Optimized *L. monocytogenes* LLO signal peptide + PEST-Codon optimized -NYESO1)
Primary amino acid sequence

M K K I M L V F I T L I L V S L P I A Q Q T E A K D A S A F N K E N
S I S S M A P P A S P P A S P K T P I E K K H A D G S Q A E G R G T
G G S T G D A D G P G G P G I P D G P G G N A G G P G E A G A T G
G R G P R G A G A A R A S G P G G G A P R G P H G G A A S G L N G
C C R C G A R G P E S R L L E F Y L A M P F A T P M E A E L A R R S
L A Q D A P P L P V P G V L L K E F T V S G N I L T I R L T A A D H
R Q L Q L S I S S C L Q Q L S L L M W I T Q C F L P V F L A Q P P S
G Q R R

FIGURE 27

Phly(10403S)-Usp45-CodOp
(330 nts.)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAGCGAATTCGCCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTAAAAAATGTAGAAGGAGA
GTGAAACCCATGAAAAAAAAAATTATTAGTGCAATTTTAATGAGTACAGTTATTTTA
AGTGCAGCAGCACCATTAAGTGGTGTTTATGCAGATACAGGATCC

FIGURE 28

Phly(10403S)-p60SP-Native
(330 nts.)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAGCGAATTCGCCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGAATATGAAAAAAGCAACTATCGCGGCTACAGCTGGGATTGCGGT
AACAGCATTTGCTGCGCCAACAATCGCATCCGCAAGCACTGGATCC

FIGURE 29

Phly(10403S)-p60SP-CodOp
330 nts.

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAAGCGAATTTGCGCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGAATATGAAAAAAGCAACAATTGCAGCAACAGCAGGTATTGCAGT
TACAGCATTTGCAGCACCAACAATTGCAAGTGCAAGTACAGGATCC

FIGURE 30

hlyP-p60 (KpnI-BamHI)

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTA
ACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAGCGAATTTCGCCAATATTATAATTATCAAA
AGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGA
GTGAAACCCATGAATATGAAAAAGCAACTATCGCGGCTACAGCTGGGATTGCGGT
AACAGCATTTGCTGCGCCAACAATCGCATCCGCAAGCACTGTAGTAGTCGAAGCTG
GTGATACTCTTTGGGGTATCGCACAAAGTAAAGGGACTACTGTTGACGCAATTA AAA
AAGCAAACAATTTAACAACAGATAAAATCGTACCAGGTCAAAAATTACAAGTAAAT
AATGAGGTTGCTGCTGCTGAAAAACAGAGAAATCTGTTAGCGCAACTTGTTAAA
CGTCCGTAGTGGCGCTGGTGTGATAACAGTATTATTACGTCCATCAAAGGTGGAAC
AAAAGTAAGTGTGAAACAACCGAATCTAACGGCTGGCACAAAATTACTTACAACG
ATGGAAAACTGGTTTCGTTAACGGTAAATACTTAACTGACAAAGCAGTAAGCACT
CCAGTTGCACCAACACAAGAAGTGAAAAAGAACTACTACTCAACAAGCTGCACC
TGCTGCAGAAACAAAACTGAAGTAAAACAAACACTACACAAGCAACTACACCTGCGC
CTAAAGTAGCAGAAACGAAAGAACTCCAGTAGTAGATCAAAATGCTACTACACAC
GCTGTTAAAAGCGGTGACACTATTTGGGCTTTATCCGTAAAATACGGTGTTTCTGTTT
AAGACATTATGTCATGGAATAATTTATCTTCTTCTTCTATTTATGTAGGTCAAAAGCT
TGCTATTAACAAACTGCTAACACAGCTACTCCAAAAGCAGAAAGTGAACCGGAAG
CTCCAGCAGCTGAAAAACAAGCAGCTCCAGTAGTTAAAGAAAATACTAACACAAAT
ACTGCTACTACAGAGAAAAAGAAACAGCAACGCAACAACAACAGCACCTAAAG
CACCAACAGAAGCTGCAAAACCAGCTCCTGCACCATCTACAAACACAAATGCTAAT
AAAACAAATACAAATACAAATACAAATACAAATACAAACAATACTAATACAAATAC
ACCATCTAAAAATACTAATACAAACTCAAATACTAATACGAATACAAACTCAAATA
CGAATGCTAATCAAGGTTCTTCCAACAATAACAGCAATTCAAGTGCAAGTGCTATTA
TTGCTGAAGCTCAAAAACACCTTGAAAAAGCTTATTTCATGGGGTGGTAACGGACCA
ACTACATTTGATTGCTCTGGTTACACTAAATATGTATTTGCTAAAGCGGGAATCTCCC
TTCCACGTACTTCTGGCGCACAAATACGCTAGCACTACAAGAATCTCTGAATCTCAAG
CAAAACCTGGTGATTAGTATTCTTTGACTATGGTAGCGGAATTTCTCACGTTGGTAT
CTACGTTGGTAATGGTCAAATGATTAAACGCGCAAGACAATGGCGTTAAATACGATA
ACATCCACGGCTCTGGCTGGGGTAAATATCTAGTTGGCTTCGGTCGCGTATAATTAA
GGATCC

FIGURE 31

FIGURE 32A

Construct: pAM401-MCS

Plasmid pAM401 containing multiple cloning site (MCS) from pPL2 vector

Insertion of small *Aat II* MCS fragment from pPL2 inserted into pAM401 plasmid between blunted *Xba I* and *Nru I* sites.

Complete pAM401-MCS plasmid sequence shown

```
CTTTAAACGTGGATCATTTTCTTTAAATTTATGCTGACGACCTTTGAATTTGCCTTTTTCTTAGCAATT
TCGATTCCTTGTGCCTGACGTTCTTAATTTTTTTCGTTCTGATTCTGCTTGATACTTGTACAATTCAAT
GACAAGGCTATTAATCAAACGCCCTTAAATTTTCATCTTCAATACCATTCATTGAGGGTAAATTTAAGAC
TTCCAGGGTTGCCCCCTTAATTTGAATTTGATTCAATTTCTGTTAATTTCTTATTATTTTCGTCCTAATC
GATCTAATTCAGTAACAATAACAATATCCCCCTTCACGAATATAGTTAAGCATAGCTTGTAAATTTGTGGGC
GTTCCGACCGATTGACCGCTTAATTTGTCTGAAAAGACCTTAGAAAACGCCCTGTAACGCTTGTAAATTGCC
GATCTAAGTTCTGTTCTTTGCTACTGACACGTGCATAACCAATTTTAGCCATTTTCAACCAACCTCTAA
AATTTCTCTCGGTTGCAATAACCAATCAGCAATATCTACTTTTTCAATTTCAAAATGCTTATCAGAAAT
GTCTTTTCGTAAGCGATAAAAATCTTGGCGCATATTGTTGCTCATTAAGAAATAGCCACCACCTTCGTCATTT
TCTAAAACTCGATAAAATAAATTTTTCATTTTACTCCTCCTATTATGCCCAACTTAAATGACCTATTCAC
CAAGTCAATTATACTGCTAAAATCATATTAGGACAAATAGGTATACTCTATTGACCTATAAATGATAG
CAACTTAAAAGATCAAGTGTTCGCTTCGCTCTCACTGCCCTCGACGTTTATAGTAGCCTTTCCCTCACTT
CGTTCAGTCCAAGCCAATAAAAGTTTTCGGGCTACTCTCTCCTTCTCCCCCTAATAATTAATTAATAAT
CTTACTCTGTATATTTCTGCTAATCATTCACATAAACAGCAAAAGAAAAACAAACACGTATCATAGATAT
AAATGTAATGGCATAGTGGGGTTTTATTTTTCAGCCTGTATCGTAGCTAAACAAATCGAGTTGTGGGTC
CGTTTTGGGGCGTTCTGCCAATTTGTTTAGAGTTTCTTGAATAAAATGTACGTTCTAAATTAACGAAGC
TGTCAGCGCCTTTATATAGCTTTCTCGTTCTTTCTTTTAAATTAATGATCGATAGCAACAATGATTTA
ACACTAGCAAGTTGAATGCCACCATTTCTCCTGGTTTAAATCTTAAAGAAAAATTCCTGATTCGCCTTC
AGTACCTTCAGCAATTTATCTAATGTCCGTTTCAAGGAATGCCTAGCACTTCTCTAATCTCTTTTTGGTCG
TCGCTAAATAAGGCTTGATACATCGCTTTTTTCGCTAATATAAGCCATTAAATCTTCTTCCATTCTGA
CAAATGAACACGTTGACGTTCCGTTCTTTTTCTTGAATTTAAACCAACCTTGACGGACAAATAAATC
TTTACTGGTTAAATCACTTGATACCCAAGCTTTGCAAAGAATGGTAATGTATCCCTATTAGCCCCTTG
ATAGTTTTCTGAATAGGCACTTCTAACAATTTTGATTACTTCTTTTCTTCTAAGGGTTGATCTAATCGA
TTATTAAACTCAAACATATTATATTTCGCACGTTTCGATTGAATAGCCTGAACTAAAAGTAGGCTAAAGA
GAGGGTAAACATAACGCTATTGCGCCCTACTAAACCCCTTTTCTCCTGAAAATTCGTTTCGTGCAATAA
GAGATTAAACCAAGGGTTCATCTACTTGTTTTTGCCTTCTGTACCGCTTAAACCCGTTAGACTTGAACG
AGTAAAGCCCTTATTATCTGTTTGTGTTGAAAGACCAATCTTGCCATTCTTTGAAAGAATAACGGTAAAT
GGGATCAAAAAATTTCTACATTGTCCGTTCTTGGTATACGAGCAATCCCAAAATGATTGCACGTTAGAT
CAACTGGCAAAGACTTTCCAAAATATTTCTGGATATTTTTCGAGATTATTTTGGCTGCTTTGACAGATT
TAAATTTCTGATTTTGAAGTCACATAGACTGGCGTTTCTAAAACAAAATATGCTTGATAACCTTTATCAG
ATTTGATAATTAACGTAGGCATAAAAACCTAAAATCAATAGCTGTTGTTAAAAATATCGCTTGCTGAAATA
GTTTCTTTTTTCCGTGTGAATATCAAAATCAATAAAGAAAGGTATTGATTGTCTTAAATTTGTTTCAGAA
TGTCCTTTAGTGTATGAACGGTTTTTCGTCTGCATACGTACCATAACGATAAACGTTTGGTGTCCAATGC
GTAAATGTATCTTGATTTTCGTGAATCGCTTCTTCGGAAGTCAGAACACGCCACGTCCGCCAATCATG
CTTTTTTTTGTAGCGATACGCAAAAATAGCCCCCTTACTTTTACCCTGGCTTGGTAGTGATTGAGCGAATT
TTACTATTTTTTAAATTTGTACTTTTAAACAAGCCGTCATGAAGCACAGTTTCTACAACAAAAGGGATATTC
ATTACGCTGTTCTCCTTTCTTACGAAAATTAATTAGTTAGAAGCTACGATCAAAGTTGAATCACAAACA
AAAAGGCAATCAACTAAGTTTTTCTAATTGATTGCCTGGTATCTTCTTAAAGACTTGAATCCCCTCA
AAAACCCGATATAATGGGTTTACAGATATTTAAGTATCTGATTAATAAAGTAATTAATACCTTTACCA
AATTTTGGGTCTCGACTTCTTTAATTGATTGGTGGTAATCAATTAAGGCTCGCAGTTAAAATTTCTCAG
GCTTTAACTGGTGGTGGCTCTTTTTTGTATTCTTTATTCAGTTTCGTTGTTTCGTTATATCTAGTATATCG
CTTTTTAAAAAAATAAGCAATGATTTTCGTGCATTATTCACACGAAATCATTCGTTTTTTCTTCTTCCATT
TCTAACTCCAATGTTACTTGTCTGTTTCTGGTTCTGGTTCTGTTGGCTCATTTGGGATTAATCCACTA
CTAGCGTTGAGTTAGTTCGCTCTAATAGCCGGTTAAGTAATAGCCGGTTAAGTGGTCAAACTTTGGG
AAAATCTCAACCCGCATTAAGTTTTGATGCCATGACAATCGTTGGAAATTTGAACAAAACATAATGCTA
AAAAGCTATCTGACTTTATGAGTGTAGAGCCACAAAATACGACTTTGGGATATACTTCAACAAAAGTTT
```

FIGURE 32B

(sequence continued from Figure 32A)

AAAGCTAAGGCACCTTCAAGAAAAAGTTTATATCGAATATGACAAAGTAAAAGCAGATACCTGGGATA
GACGTAATATGCGTGTGAATTTAATCCCAATAAACTCACACATGAAGAAATGATTTGGTTAAAAACAA
AATATTATCGACTACATGGAAGATGACGGTTTTACAAGATTAGACTTAGCTTTTGATTTTGAAGATGAT
TTGAGCGATTACTATGCAATGACTGATAAAGCAGTTAAGAAAACTGTTTTTATGGTCGTAATGGCAA
GCCAGAAAAAAAATATTTTGGTGTCCGTGATAGTGATAGATTTATTAGAATTTATAATAAAAAACAAG
AACGTAAAGATAACGCAGATGTTGAAGTTGTGTTGAACATTTATGGCGTGTAAGAAGTTGAATTAATA
AGAGATATGGTTGATTACTGGAATGATTGTTTTAATGATTTACACATCTTTGAAACCTGCGTGGGCTAC
TTTAGAAAAAATTAATGAGCAAGCTATGGTTTATACTTTGTTGCATGAAGAAAGTATGTGGGGAAAGC
TAAGTAAGAATACTAAGACTAAATTTAAAAAATTGATTAGAGAAATATCTCCAATTGATTTAACGGAA
TTAATGAAA'CGACTTTAAAAAGCGAACGAAAAACAATTGCAAAAGCAGATTGATTTTTGGCAACGTGA
ATTTAGGTTTTTGAAGTAAAAATAAGTTTATTTGATAAAAAATTGCTAATTCAGTATAATTAATTTTAC
GAGGTGACATAACGTATGAAAAAATCAGAGGATTATTCCTCCTAAATATAAAAAATTTAAAAATTTAGGA
GGAAGTTATATATGACTTTTAATATTAATTGAATTAGAAAAATTGGGATAGAAAAGAATATTTTGAACAC
TATTTTAATCAGCAAACTACTTATAGCATTACTAAAGAAATTGATATTACTTTGTTTAAAGATATGATA
AAAAAGAAAGGATATGAAATTTATCCCTCTTTAATTTATGCAATTATGGAAGTTGTAATAAAAAATAA
AGTGTTTAGAACAGGAATTAATAGTGAGAATAAATAGGTTATTGGGATAAGTTAAATCCCTTTGTATA
CAGTTTTTAATAAGCAAACTGAAAAATTTACTAACATTTGGACTGAATCTGATAAAAACTTCATTTCTT
TTTATAATAATTATAAAAAATGACTTGCTTGAATATAAAGATAAAGAAGAAATGTTTCTAAAAAACCG
ATACCTGAAAAACCCATACCGATTCAATGATTCCCTGGATTGATTTTAGTTCATTTAATTTAAATATT
GGTAACAATAGCAGCTTTTTATTGCCTATTATTACGATAGGTAAATTTTATAGTGAGAATAATAAAATT
TATATACCAGTTGCTCTGCAACTTCATCATTCTGTATGTGATGGTTACCATGCTTCACTATTATGAATG
AATTTCAAGATATAATTCATAGGGTAGATGATTGGATTTAGTTTTAGATTTTGAAAGTGAATTTAATT
TTATACACGTAAGTGATCATAAAATTTATGAACGTATAACAACCACATTTTTTGGTTGCTTGTGGTTTT
GATTTTGAATTTGGTTTTGAACTTATGGACTGATTTATTCAGTCCATTTTTTGTGCTTGCACAAAAACTA
GCCTCGCAGAGCACGCGATTAATGACTTATGAAACGTAGTAAATAAGTCTAGTGTGTTATACTTTACT
TGGAAAGATGCACCGAATAAAAAAATATTGAAGAACAACCTAGCAAAAGATTTTAAAGAGTTATTTTATT
TAAGTCTTTATAACATGAGTGAAGCGAATTTTTAAATTTTCGATAGAAATTTTTACATCAAAAAAGCCCC
TGTCAAAATTTGACGAAGGGGGTTTTTGGCGCACGCTTTTCGTTAGAAATATACAAGATTGAAATCG
TGATAAGTGCGCCCTTTGTTTTGAACTTAGCAGTTACATCAATTTTTTAAAAATGATGTATAAGTGCG
CCCTTTTAAATTTTGAGTGATTATTTTTTGAAGTTAGAAAAAGGGATTGGGAAAAATTTCCCAAAATAA
TTTAAAAAATAAGCAAAAAATTTTCGATAGAGAATGTGCTATTTTTTGTCAAAGGTGTATACCTTGACTG
TGCTTGCTGTTACATTAAGTTTATTTTTAAGTTATTAAAAAAGAAATAGCTTTTAAAGTTTGGCTCGCT
GTCGCTTTATAAAGCTGATTGACTTTTGATTGCAAACTACTTAAAGAAAAACAACCTCGGACTATTGCTT
TTCTTCTCTTTGGTTTGAACATCAGCAATTATCCCCTCTTGATTGCCTATTTTAGCTTGTTTAGAAGAA
CAAAAGCTAAAAAGCTCCTCTTGGGTTTTAAAAACGCTGTGTGGGGCTTAGAACGCCCTTAAACGACCCT
TGGTTTACTTTTATACTAGCTTCCACCTCGAAAAAAGGTTCTTTTAAAAATTTCTCTATGGCTTCTGGC
GCTGAAAAAATAAGGTATAAGGTGGGCGTTTGAACACGTCCTAGTGAAGTGTACCTTGTACGCCCCCT
TCTGTTGTAAATTTAACGTATACAAAGGGCTTGCGTTTCATGCCGATCAACCAATCGGCAATTTGGCGTG
TTTGCGCTTCTTGATAAAAGGGATAGTAATTCATTCAGGTTGCAAAATTTTGAAGCCGCTTCGGATT
CATCTTTTTCTAAGCTATTGATCCATAGTCTTTTAAATGTTTTATCTTTTGAAGGCAATTTGCTTTATG
GATAATCGACCAGGCGATATTTTACCTTCTCTGTGCTATCTGTTGCAACAATAATTGTATTGCTTTT
TTGAGAAGTTCTGCAACAATTTTAAACTGCTTTCCCTTATCTTTTGCAACTTCAAAATCGTATCGATCA
GGAAAAATCGGCAAGATTCAAGTTTCCAATTTTGCCACTTTTCGTCATAATGACCTGGTTCTGCTAAT
TCCACTAAATGCCCAAAACCAAGGTGATAAACGTTTTCATCTGTAATAGTGGGTCTTTGATCTCAAA
ATAACCGTCTTTTTTGGTGCTTTGTTTTAAAGCACTTGGTAGGCTAATGCCTGGCTGGTTTTTTCAGCT
AAAAATAACCGTACTCATTAACCTATCCCTCTTTTCAATTGTTTTTCTTTGATCGACTGTCACGTTATATCT
TGCTCGATACCTTCTAAACGTTTCGGCGATTGATTCCAGTTTGTCTTCAACTTCTTTATCGGATAAACCA
TTCAAAAAACAAATCGAAAGCATGGATGCGCCGCTGCGGCTGCTGGAGATGGCGGACGCGATGGATA
TGTTCTGCCAAGGGTTGGTTTGGCGATTACAGTTCTCCGCAAGAATTGATTGGCTCCAATTTCTGGAG
TGGTGAATCCGTTAGCGAGGTGCGCGGCTTCCATTAGGTCAGGTCAGGTCGCGCGGCTCCATGCACCGC
GACGCAACGCGGGGAGGCAGACAAGGTATAGGGCGGCGCTACAATCCATGCCAACCCGTTCCATGT
GCTCGCCGAGGCGGCATAAATCGCCGTGACGATCAGCGGTCCAGTGATCGAAGTTAGGCTGGTAAGA

FIGURE 32C

(sequence continued from Figure 32B)

GCCGCGAGCGATCCTTGAAGCTGTCCCTGATGGTCGTACCTACCTGCCTGGACAGCATGGCCTGCAA
CGCGGGCATCCCGATGCCGCGGAAGCGAGAAGAATCATAATGGGGAAGGCCATCCAGCCTCGCGTC
GCAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCTCGAGGTGACGGTATCGATAAGC
TTGATATCGAATTCCTGCAGCCCGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC
CAGCTTTTGTTCCTTTAGTGAGGGTTAATGCTAGAAATATTTTATCTGATTAATAAGATGATCTTCTTG
AGATCGTTTTGGTCTGCGCGTAATCTCTTGCTCTGAAAACGAAAAAACCGCCTTGACGGGCGGTTTTTC
GAAGGTTCTCTGAGCTACCAACCTCTTGAACCGAGGTAACCTGGCTTGGAGGAGCGCAGTCACCAAAAC
TTGTCTTTCACTTTAGCCTTAACCGGGCGCATGACTTCAAGACTAACTCCTCTAAATCAATTACCACTG
GCTGCTGCCAGTGGTGTCTTTGCATGTCTTTCCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG
CAGCGGTGCGACTGAACGGGGGGTTCGTGCATACAGTCCAGCTTGGAGCGAACTGCCTACCGGAACT
GAGTGTACGGCGTGGAATGAGACAAACCGGGCCATAACAGCGGAATGACACCGGTAACCGGAAAGGC
AGGAACAGGAGAGCGCACGAGGGAGCCGCCAGGGGGAAACGCCTGGTATCTTTATAGTCTGTGCGG
TTTCGCCACCACTGATTTGAGCGTCAGATTTCCGTGATGCTTGTACGGGGGGCGGAGCCTATGGAAAAA
CGGCTTTGCCGCGGCCCTCTCACTTCCCTGTAAAGTATCTTCTGGCATCTTCCAGGAAATCTCCGCCCC
GTTCTGAAGCCATTTCCGCTCGCCGACGTGCAACGACCGAGCGTAGCGAGTCACTGAGCGAGGAAGC
GGAATATATCCTGTATCACATATTTCTGCTGACGCACCGGTGCAGCCTTTTTTCTCTGCCACATGAAGC
ACTTCACTGACACCCTCATGAGTCCCAACATAGTAAGCCAGTATACACTCCGCTAGCGCTGATGTCCG
GCGGTGCTTTTGCCGTACGCACCAACCCCGTCAGTAGCTGAAACAGGAGGGACAGCTGATAGAAACAGA
AGCCACTGGAGCACCTCAAAAACACCATCATACACTAAATCAGTAAGTTGGCAGCATACCCGACGCA
CTTTGCGCCGAATAAATACCTGTGACGGAAGATCACTTCGCAGAATAAATAAATCCTGGTGTCCCTGT
TGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAAATGAGACGTTGATCGGCACGTAAGAGGTTCCA
ACTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTGAGTTATCGAGATTTTCAGGAGCTA
AGGAAGCTAAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCATCGTAA
AGAACATTTGAGGCATTTCACTGAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTAC
GGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAGTTTATCCGGCCTTTATTCACATTCTTGCCCG
CCTGATGAATGCTCATCCGGAATTCGCTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTG
TTCACCCTTGTACACCGTTTTCCATGAGCAAACTGAAACGTTTTTCATCGCTCTGGAGTGAATACCACG
ACGATTTCCGGCAGTTTCTACACATATATTGCAAGATGTGGCGTGTACGGTGAAACCTGGCCTATT
TCCCTAAAGGGTTTATTGAGAATATGTTTTTCGCTCTAGCCAATCCCTGGGTGAGTTTACCAGTTTTG
ATTTAAACGTGGCCAATATGGACAACCTCTTCGCCCCCGTTTTCAACATGGGCAAATATTATACGCAAG
GCGACAAGGTGCTGATGCCGCTGGCGATTACAGGTTTCATCATGCCGTCTGTGATGGCTTCCATGTCCGC
AGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGCGTAATTTTTTAAGGCAG
TTATTGGTGCCCTTAAACCGCTGGTGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAA
TTCGAAAGCAAATTCGACCCGGTTCGTTCGGTTTCAGGGCAGGGTCGTTAAATAGCCGCTTATGTCTATTG
CTGGTTTACCGGTTTATTGACTACCGGAAGCAGTGTGACCGTGTGCTTCTCAAATGCCTGAGGCCAGTT
TGCTCAGGCTCTCCCCGTGGAGGTAATAATTGACGATATGATCATTTATTCTGCCTCCAGAGCCTGAT
AAAAACGGTTAGCGCTTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGAT
GCAGATCCGGAACATAATGGTGCAGGGCGCTTGTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCG
GGGACTGTTGGGCGCTGCCGGCACCTGTCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCG
GCGACGATAGTCATGCCCGCGGCCACCGGAAGGAGCTACCGGACAGCGGTGCGGACTGTTGTAACCT
AGAATAAGAAATGAGGCCGCTCATGGCGTTGACTCTCAGTCATAGTATCGTGGTATCACCGGTTGGTT
CCACTCTCTGTTGCGGGCAACTTCAGCAGCACGTAGGGGACTTCCGCGTTTCCAGACTTACGAAACA
CGGAAACCGGAAGACCATTCATGTTGTTGCTCAGGTGCGCAGACGTTTTGCAGCAGCAGTCGCTTCACGT
TCGCTCGCGTATCGGTGATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCTCA
ACGACAGGAGCAGCATCATGCGCACCCGTGGCCAGGACCCAACGCTGCCCGA

Human Mesothelin Gene
Codon-Optimized for Expression in Listeria

ATGGCATTGCCAACTGCACGTCCATTACTAGGTAGTTGCGGTACACCAGCACTAGGT
TCTTTATTATTTTTGTTATTTTCTCTAGGTTGGGTTCAACCAAGTCGTACATTAGCAG
GTGAAACAGGTCAAGAAGCAGCACCACCTTGACGGTGTATTAACGAATCCACCAAAT
ATATCAAGTTTAAAGTCCACGTCAATTATTAGGTTTTCCATGTGCAGAAAGTTTCAGGTT
TAAGTACAGAACGTGTCCGTGAGTTAGCAGTTGCATTAGCACAAAAAACGTTAAA
TTATCTACAGAACAGTTACGTTGTTTAGCCCATAGATTAAGCGAACCACCAGAAGAC
TTAGATGCACTTCCTTTAGACCTTCTTTTATTCTTAAATCCAGATGCATTTTCAGGAC
CACAAGCATGTACACGTTTTTTTAGTCGAATTACAAAAGCCAATGTTGATTTATTAC
CTCGTGGGGCTCCTGAAAGACAACGTTTATTACCTGCTGCATTAGCATGCTGGGGTG
TTCGCGGTAGCTTATTAAGTGAAGCCGATGTTTCGTGCTTTAGGGGGTTTAGCATGTG
ATTTACCTGGTCGTTTCGTTGCAGAATCAGCAGAAGTGTTATTACCGAGATTAGTTTC
ATGCCCAGGACCTTTAGATCAAGATCAACAAGAGGCAGCTAGAGCAGCTCTTCAAG
GAGGAGGCCACCATATGGCCCACCAAGTACATGGAGTGTTTCTACAATGGATGCG
TTAAGAGGTTTATTACCGGTTTTAGGACAACCAATTATTCGTAGTATTCCACAAGGC
ATTGTAGCAGCATGGCGTCAACGTAGTTCTCGTGATCCGTCTTGGCGACAACCAGAA
CGTACAATTCTACGTCCAAGATTTTCGTAGAGAAAGTAGAAAAAACGGCGTGTCTAGT
GGCAAAAAGCACGTGAAATTGATGAAAGTTTAAATTTTTATAAAAAATGGGAATT
AGAAGCATGTGTGCATGCAGCATTACTAGCTACACAAATGGATCGTGTTAATGCTAT
TCCATTCACATATGAACAATTAGATGTTTTAAAGCATAAATTAGACGAATTATATCC
ACAAGGTTATCCAGAATCAGTTATTCAACATTTAGGTTACTTATTTTTAAAAATGAG
TCCAGAAGACATACGCAAATGGAATGTTACAAGTTTAGAAACATTAAAAGCGCTTTT
AGAAGTTAACAAGGTCATGAAATGAGTCCACAAGTTGCTACGTTAATTGATAGATT
CGTTAAAGGCCGTGGTCAATTAGATAAAGATACTTTAGATACATTAAACAGCATTTTA
TCCTGGCTACTTATGCAGTTTATCACCAGAAGAATTAAGTTCCGTTCCACCGAGTAG
TATCTGGGCAGTTCGTCCGCAAGATTTAGATACATGCGACCCACGTCAATTAGATGT
TTTATATCCAAAAGCAAGATTAGCTTTCCAAAATATGAACGGTAGTGAATATTTTCGT
AAAAATTCAATCCTTTTTAGGTGGTGCACCAACTGAAGATCTAAAAGCATTAAAGCCA
ACAAAATGTAAGTATGGATTTAGCTACGTTTATGAAATTACGTACAGATGCAGTTCT
ACCATTAACAGTTGCAGAAGTTCAAAAATTATTAGGTCCACACGTAGAAGGATTAA
AAGCAGAAGAACGTCACCGTCCAGTTCGCGATTGGATTTTACGTCAACGTCAAGATG
ATTTAGATACATTAGGTTTAGGTTTACAAGGCGGTATTCCGAATGGATATTTAGTGT
TAGATTTATCTGTTCAAGAAGCATTAAAGTGGTACACCGTGTTTATTAGGTCCAGGTC
CAGTTTTAACAGTGTTAGCATTATTATTAGCCAGTACATTAGCTTAA

FIGURE 33

MALPTARPLLGSCTPALGSLLFLLFSLGWVQPS
RTLAGE TGQEAAPLDGVLTNPPNISSLSPRQLLG
FPCA EVSGLSTERVRELAVALAQKNVKLSTEQLR
CLAHRLSEPPEDLDALPLDLLLFLNPDAFSGPQA
CTRFFSRITKANVDLLPRGAPERQRLLPAAALACW
GVRGSLLSEADVRA LGGLACDLPGRFVAESA EV
LLPRLVSCPGPLDQDQGEAARAALQGGGGPPYGGP
STWSVSTMDALRGLLPVLGQPIIRSIPQGIVAAW
RQRSSRDPSWRQPERTILRPRFRREVEKTACPSG
KKAREIDESLIFYKKWELEACVDAALLATQMDR
VNAIPFTYEQLDVLKHKLDELYPQGYPESVIQHL
GYLFLKMSPEDIRKWNVTSLET LKALLEVNKGHE
MSPQVATLIDRFVKGRGQLDKDTLDTLTAFYPG
YLCSLSPEELSSVPPSSIWAVRPQDLDTCDPRQL
DVLYPKARLAFQNMNGSEYFVKIQSFLGGAPTED
LKA LSQQNVSMDLATFMKLRTDAVLPLTVAEVQ
KLLGPHVEGLKAEERHRPV RDWILRQRQDDLDLDT
LGLGLQGGIPNGYLVLDLSVQEALSGTPCLLGPG
PVLTVLALLLASTLA

FIGURE 34

Murine Mesothelin Gene
Codon-Optimized for Expression in *Listeria*

ATGGCATTACCAACGGCTCGCCCATATTAGGTTCTTGTGGTTCACCAATTTGTAGTC
GCAGTTTTTTATTATTACTATCTTTAGGTTGGATTCCGCGTTTACAAACACAAAC
CACTAAAACAAGTCAAGAAGCTACATTATTGCATGCAGTCAATGGCGCAGCAGATT
TTGCAAGTTTACCAACAGGCTTATTTCTTGGTCTTACATGTGAAGAAGTTAGTGATTT
AAGTATGGAACAAGCAAAAGGTTTAGCGATGGCGGTTCCGCAAAAAAATATTACAT
TACGTGGTCATCAATTACGTTGTTTAGCACGTCGTTTACCACGACATTTAACAGATG
AAGAATTTAAATGCTCTACCATTAGACTTATTATTATTTTAAATCCAGCAATGTTTCC
AGGTCAACAAGCATGTGCCCATTTTTTCAGTTTAATTTTCGAAAGCAAATGTAGATGT
TTTACCGAGACGTAGCTTAGAACGTCAACGTCTTTTAATGGAAGCATTAAAAATGTCA
AGGTGTTTATGGTTTCCAAGTTAGTGAAGCAGATGTTCTGTGCACTTGGTGGTTTACG
TTGTGATTTACCAGGGAAATTTGTAGCACGTTCTAGTGAAGTATTATTACCATGGTT
AGCAGGTTGTCAAGGTCCATTAGATCAAAGTCAAGAAAAAGCAGTTCGTGAAGTCT
TACGTAGTGGTCGTAATATGGCCACCTAGCAAATGGAGTGTTAGTACGTTAG
ATGCATTACAAAGTTTAGTAGCTGTTTTAGATGAAAGTATTGTTTACAGATATTCCAA
AAGATGTGAAAGCAGAGTGGTTACAACATATTTCCCGTGACCCATCTCGTTTAGGTA
GTAAATTAACAGTTATTCATCCACGTTTTTCGCCGCGACGCAGAACAAAAAGCATGTC
CACCAGGTAAAGAACCATATAAAGTAGATGAAGATTTAATTTTTTATCAGAATTGGG
AATTAGAAGCCTGTGTGATGGTACAATGTAGCACGTCAAATGGATTTAGTTAATG
AAATTCATTTACATATGAACAATTAAGTATCTTTAAACATAAATTAGATAAAACAT
ATCCACAAGGTTATCCAGAATCGTTAATTCAACAATTAGGTCAATTTTTTCGTTATGT
TAGTCCAGAAGACATTCATCAATGGAATGTTACAAGTCCAGATACAGTTAAAACTTT
ATTAAGGTTAGTAAAGGTCAAAAAATGAATGCTCAAGCAATTGCATTAGTCCGAT
GTTATTTACGTGGAGGTGGTCAATTAGATGAAGATATGGTTAAAGCATTAGGGGATA
TTCATTATCATATTTATGTGATTTCTCCCCACAAGACTTACATTCAGTTCCAAGTAG
TGTTATGTGGTTAGTTGGTCCACAAGGTTTAGATAAATGTAGTCAACGTCATTTAGG
TTTACTTTATCAAAAAGCATGTAGTGCGTTTCAAAATGTTAGTGGTTTAGAATATTTT
GAAAAAATCAAAACATTTTATAGGAGGTGCATCTGTAAAAGATTTACGCGCATTAAGT
CAACATAATGTAAGTATGGATATCGCAACATTTAAACGTTTACAAGTCGATAGTCTA
GTTGGTCTTAGTGTAGCAGAAGTTCAAAAATTATTAGGGCCGAATATTGTAGATTTA
AAACAGAAGAAGATAAAAGTCCAGTTCGTGACTGGTTATTTGACAACATCAGAA
AGACTTAGATCGTCTTGGATTAGGTTTACAAGGTGGTATTCCAAATGGTTATTTAGTT
TTAGATTTTAATGTACGTGAAGCATTTAGTTCAAGAGCGAGTTTATTAGGTCCAGGT
TTTGTGTTAATTTGGATTCCAGCATTACTACCAGCACTTCGTTTATCATAA

FIGURE 35

Murine Mesothelin Primary Amino Acid Sequence

M A L P T A R P L L G S C G S P I C S R S F L L L L L S L G W I P R L
Q T Q T T K T S Q E A T L L H A V N G A A D F A S L P T G L F L G L
T C E E V S D L S M E Q A K G L A M A V R Q K N I T L R G H Q L R
C L A R R L P R H L T D E E L N A L P L D L L L F L N P A M F P G Q
Q A C A H F F S L I S K A N V D V L P R R S L E R Q R L L M E A L K
C Q G V Y G F Q V S E A D V R A L G G L A C D L P G K F V A R S S
E V L L P W L A G C Q G P L D Q S Q E K A V R E V L R S G R T Q Y
G P P S K W S V S T L D A L Q S L V A V L D E S I V Q S I P K D V K
A E W L Q H I S R D P S R L G S K L T V I H P R F R R D A E Q K A C
P P G K E P Y K V D E D L I F Y Q N W E L E A C V D G T M L A R Q
M D L V N E I P F T Y E Q L S I F K H K L D K T Y P Q G Y P E S L I
Q Q L G H F F R Y V S P E D I H Q W N V T S P D T V K T L L K V S K
G Q K M N A Q A I A L V A C Y L R G G G Q L D E D M V K A L G D I
P L S Y L C D F S P Q D L H S V P S S V M W L V G P Q G L D K C S Q
R H L G L L Y Q K A C S A F Q N V S G L E Y F E K I K T F L G G A S
V K D L R A L S Q H N V S M D I A T F K R L Q V D S L V G L S V A
E V Q K L L G P N I V D L K T E E D K S P V R D W L F R Q H Q K D
L D R L G L G L Q G G I P N G Y L V L D F N V R E A F S S R A S L L
G P G F V L I W I P A L L P A L R L S

FIGURE 36

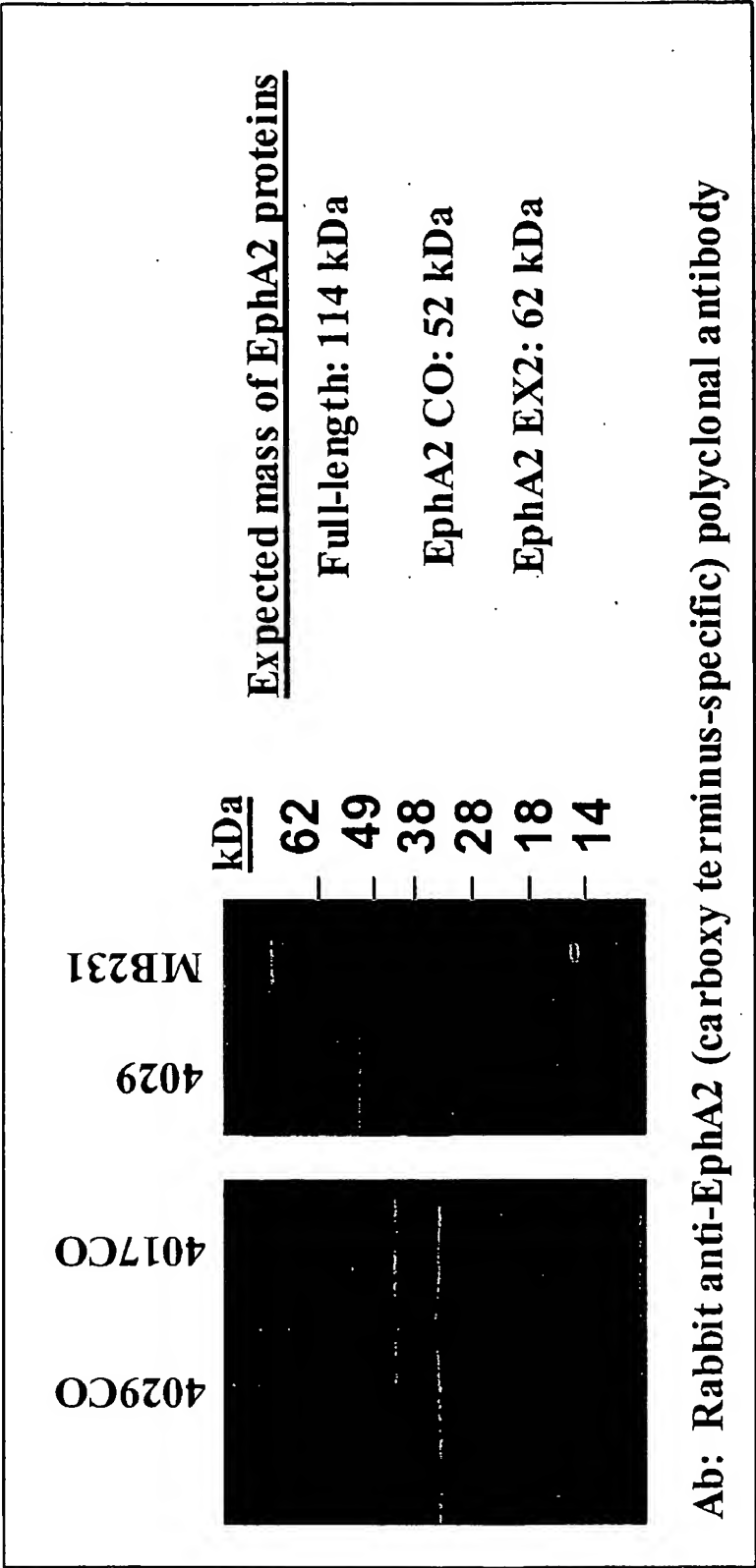


FIGURE 37

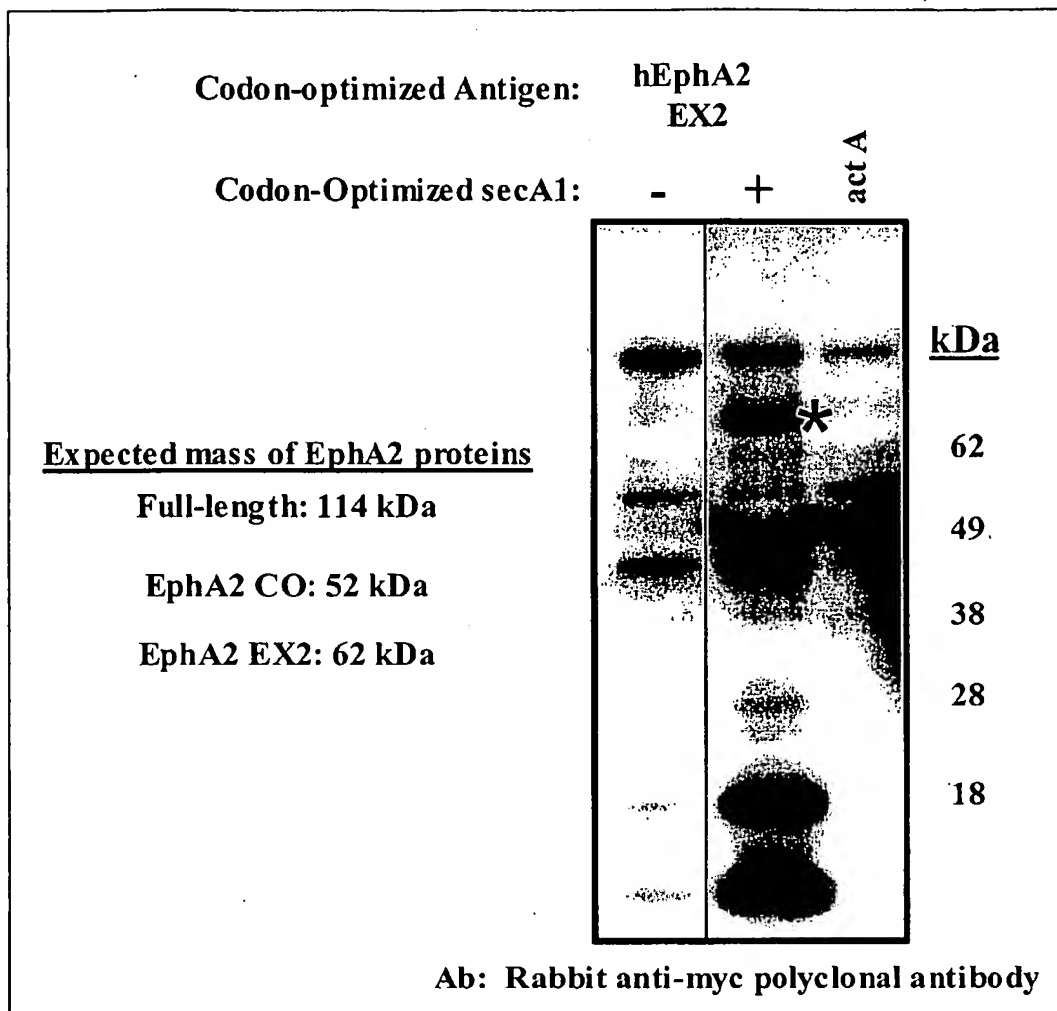


FIGURE 38

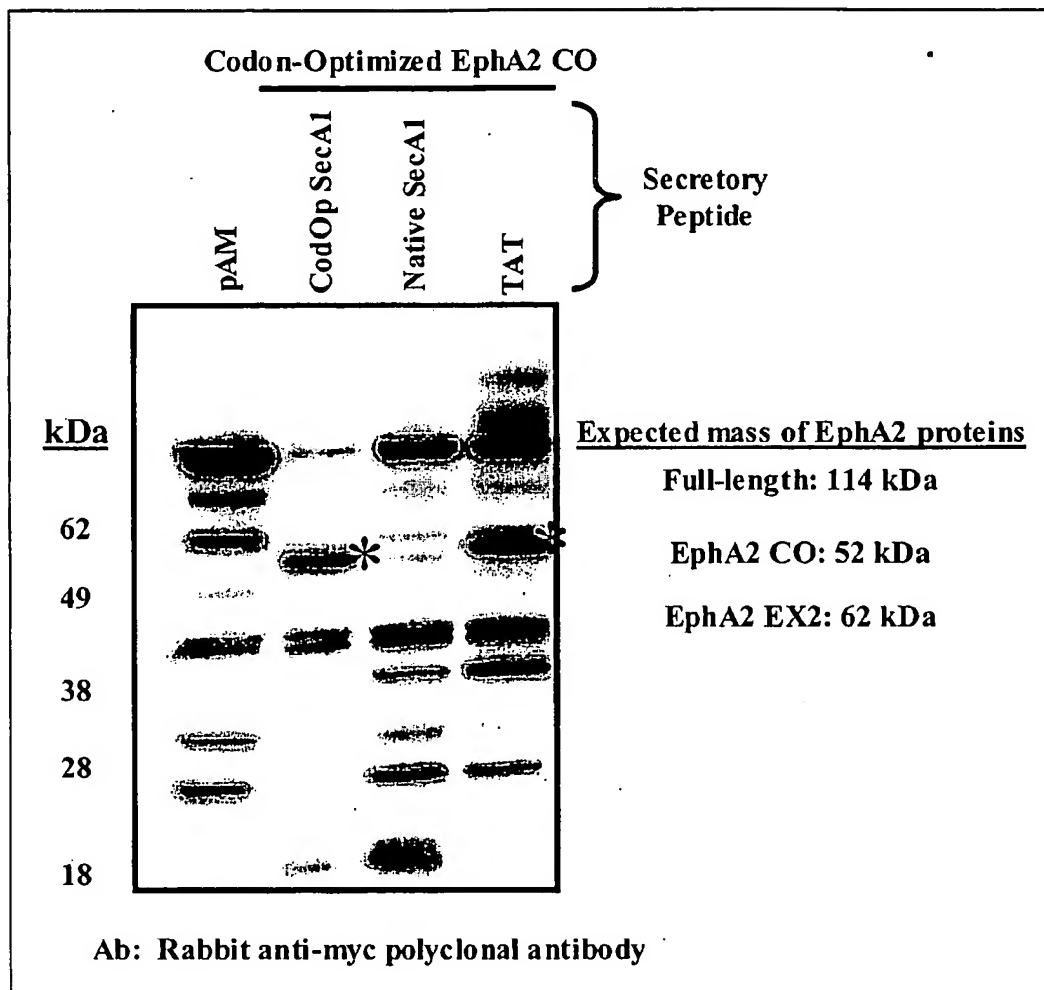


FIGURE 39

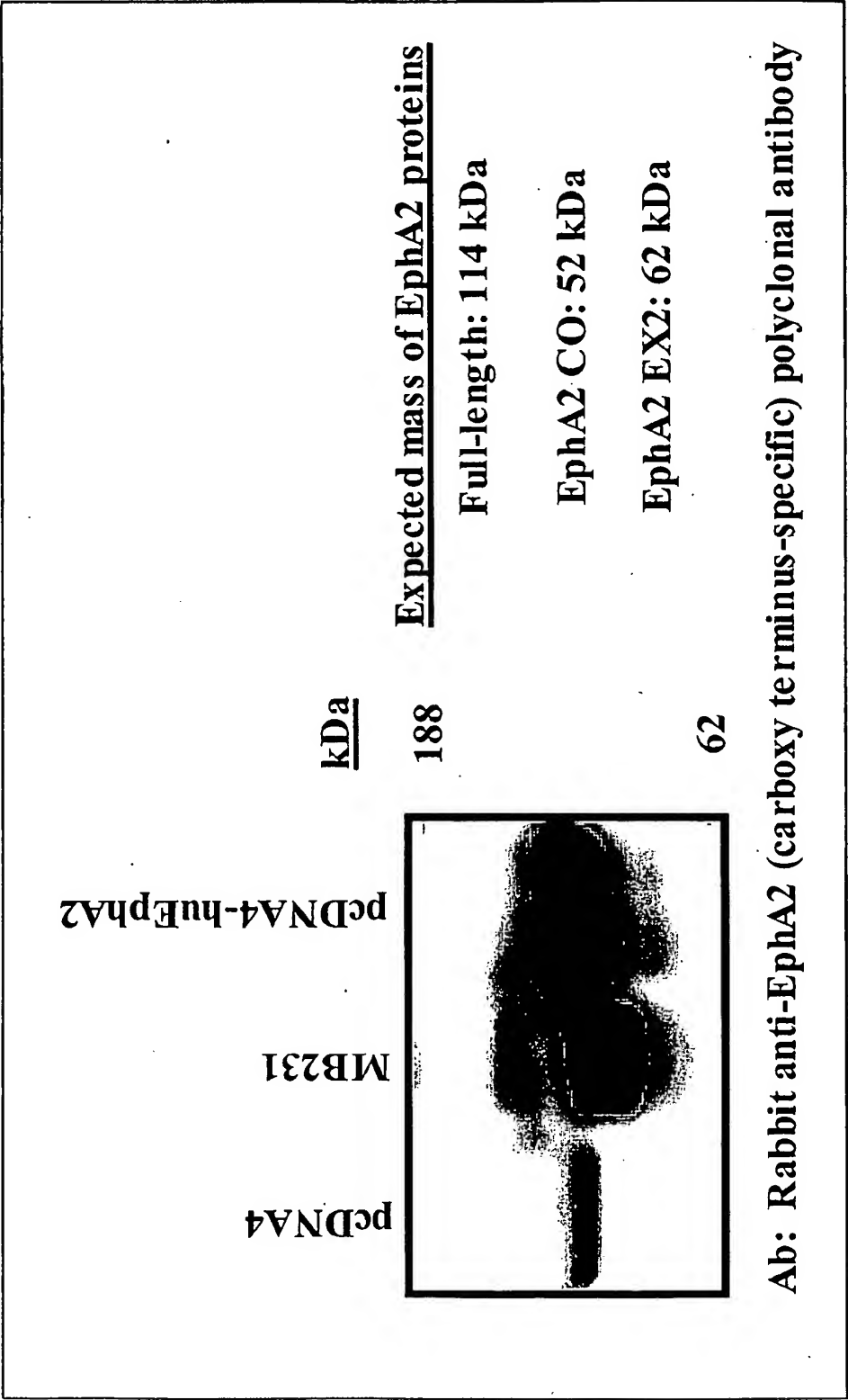


FIGURE 40

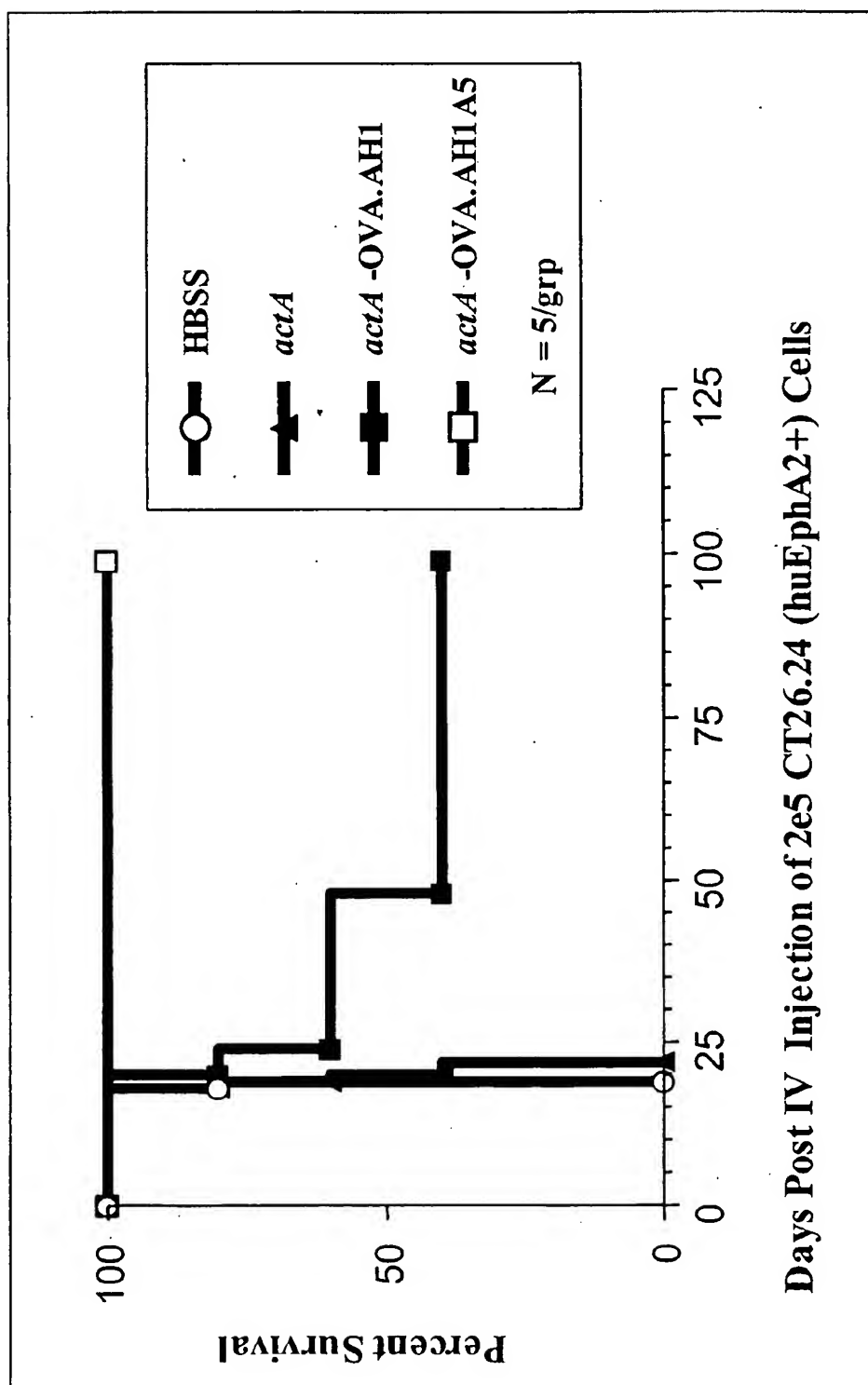


FIGURE 41

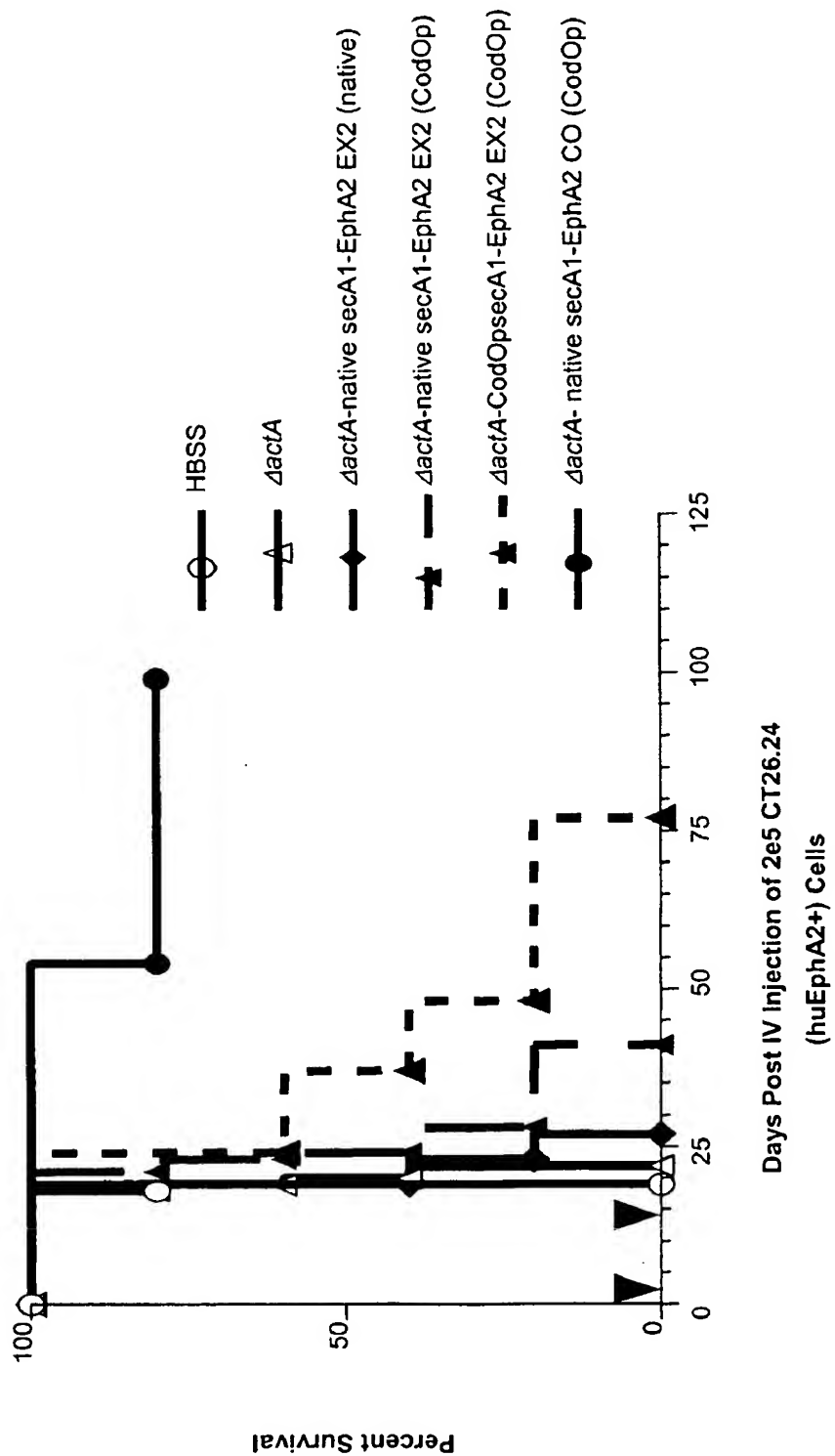


FIGURE 42

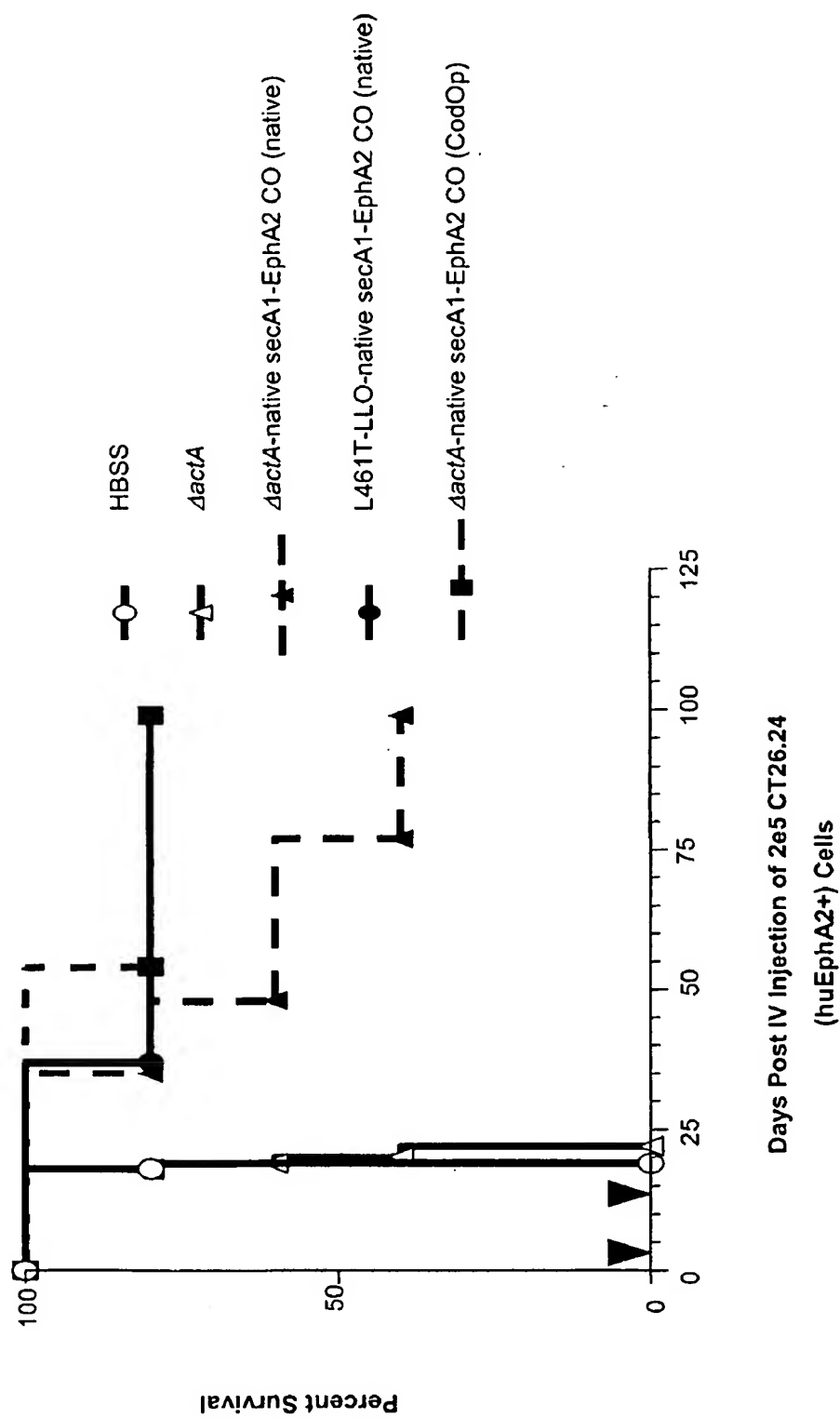


FIGURE 43

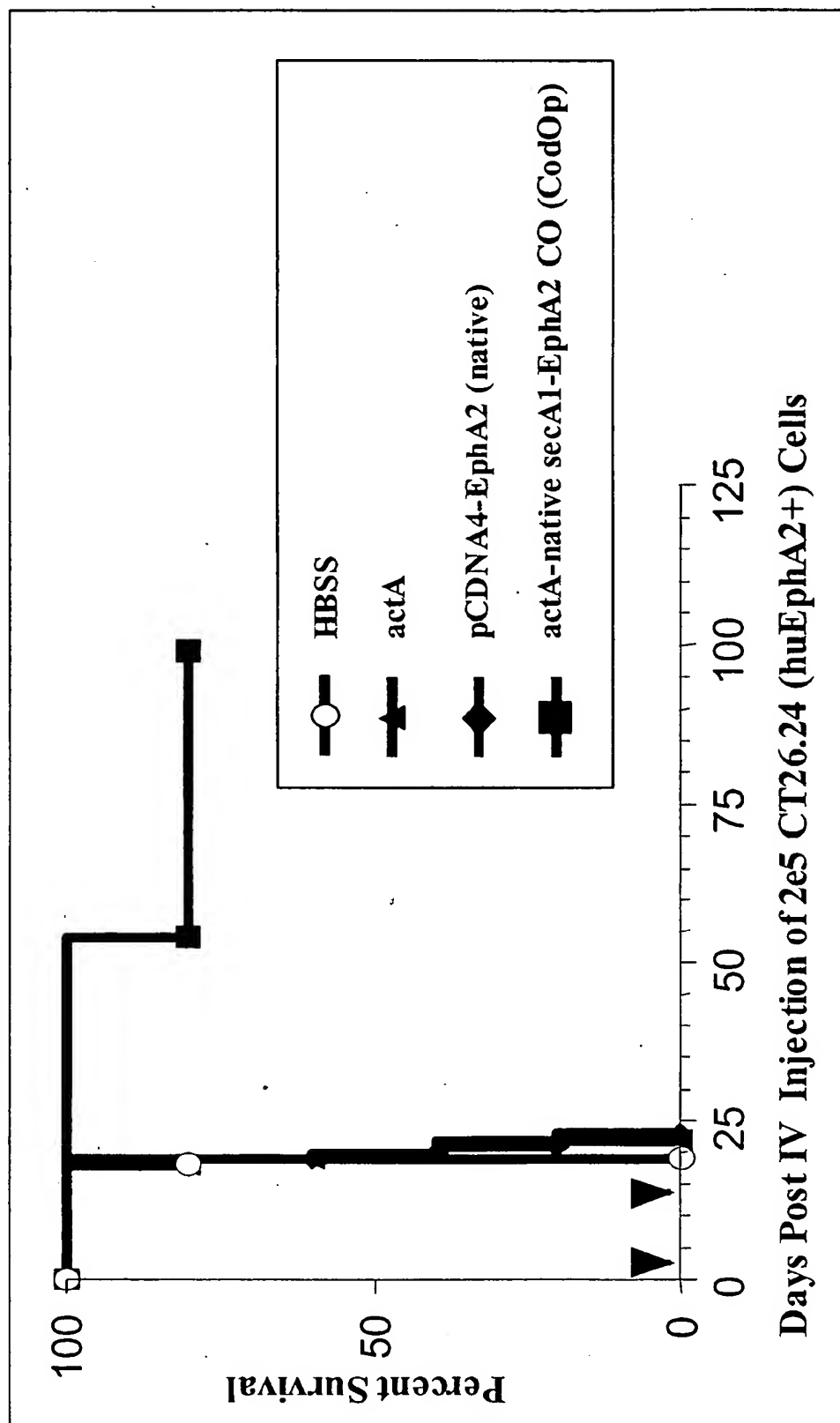


FIGURE 44

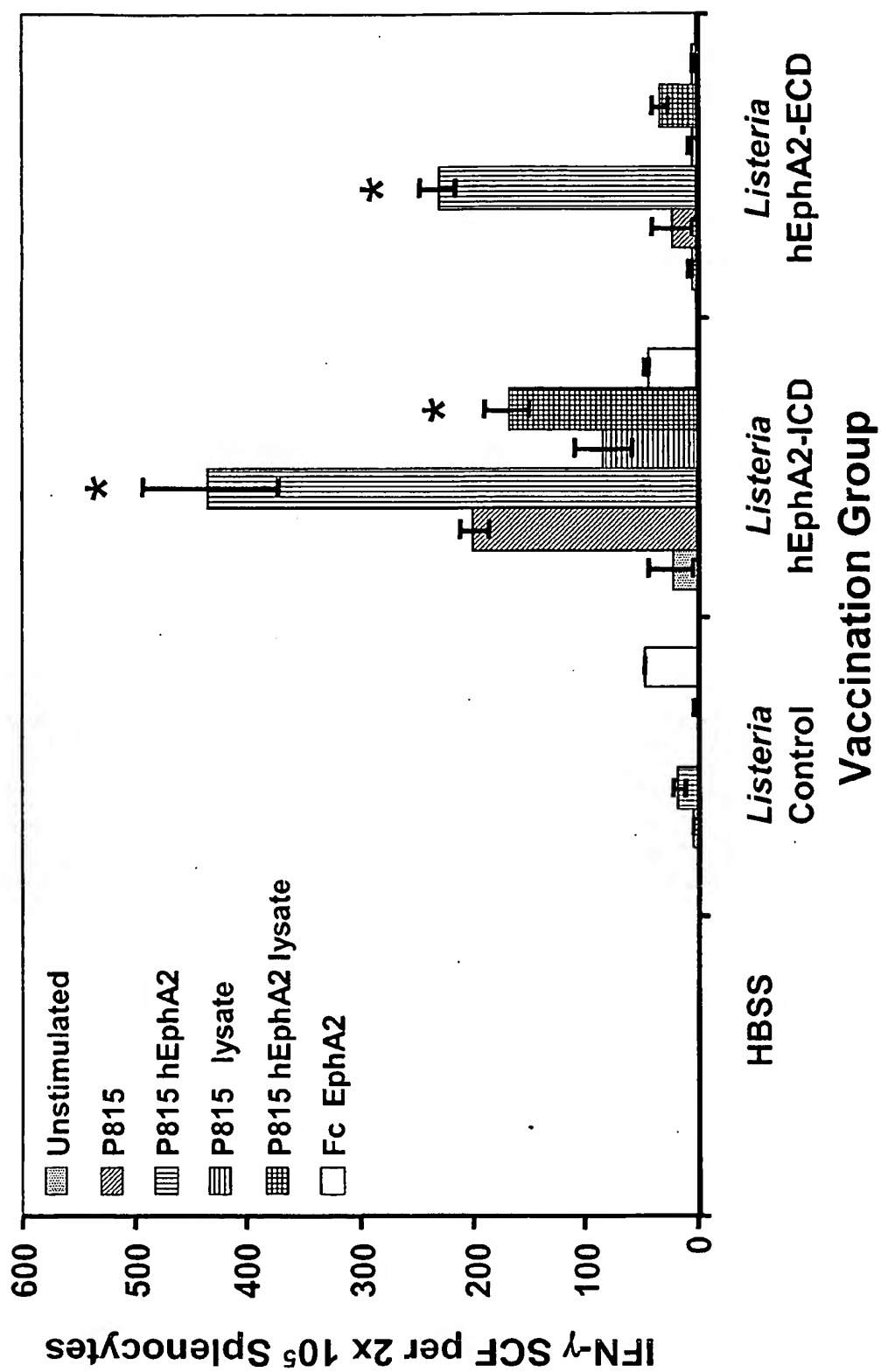


FIGURE 45

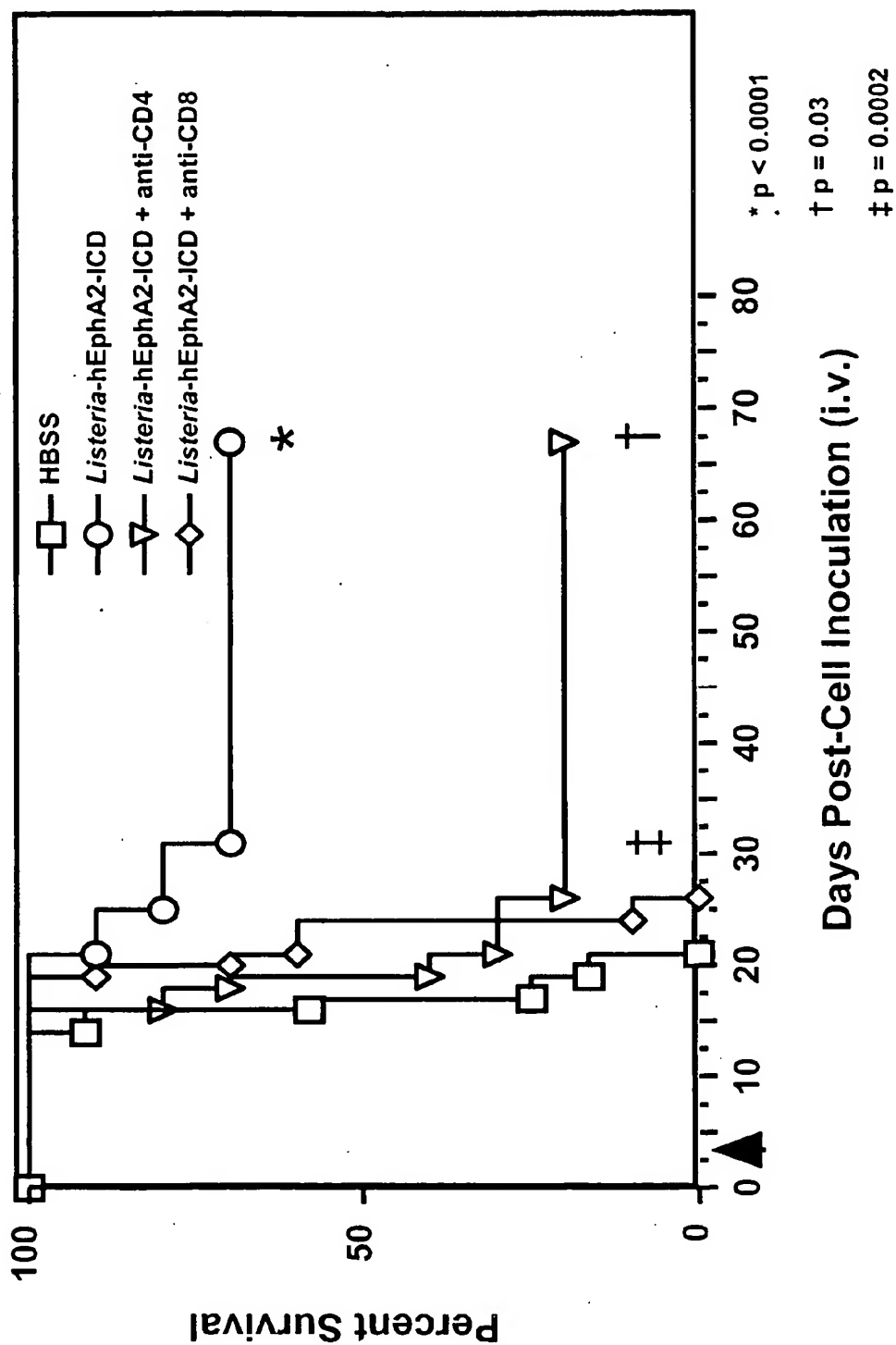
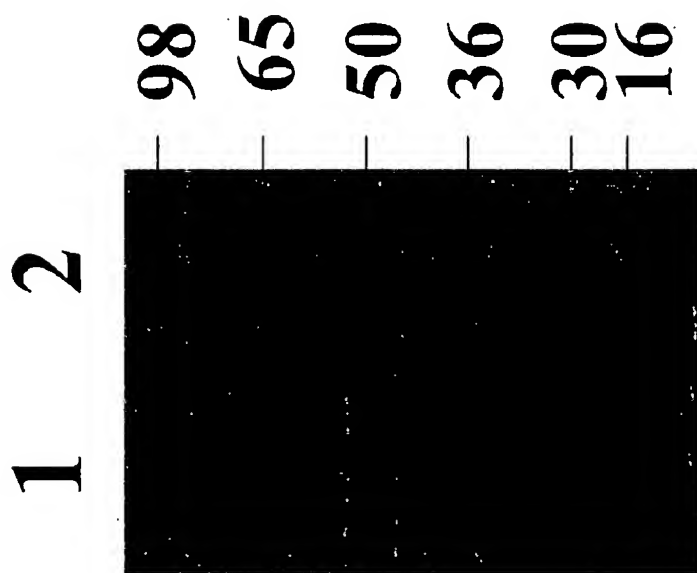


FIGURE 46

hly promoter-codon optimized Ba PA signal peptide sequence
(Unique 5' and 3' *Kpn I* and *Bam HI* sites underlined)

GGTACCTCCTTTGATTAGTATAATTCCTATCTTAAAGTTACTTTTATGTGGA
GGCATTAAACATTTGTTAATGACGTCAAAGGATAGCAAGACTAGAAATAAA
GCTATAAGCAAGCATATAATTTGCGTTTCATCTTTAGAACGGAATTTC
GCCAATATTATAATTATCAAAAGAGAGGGTGGCAACGGTATTTTGGCAT
TATTAGGTTAAAAAATGTAGAAGGAGAGTGAAACCCATGAAAAAACGTA
AAGTTTAAATTCATTAAATGGCATTAAAGTACAATTTTAGTTAGTAGTACAG
GTAATTTAGAAAGTTATTCAAGCAGAAAGTTGGATCC

FIGURE 47



LEGEND
 1. $\Delta actA$ -LLO/hMeso (native)
 2. $\Delta actA$

FIGURE 48A

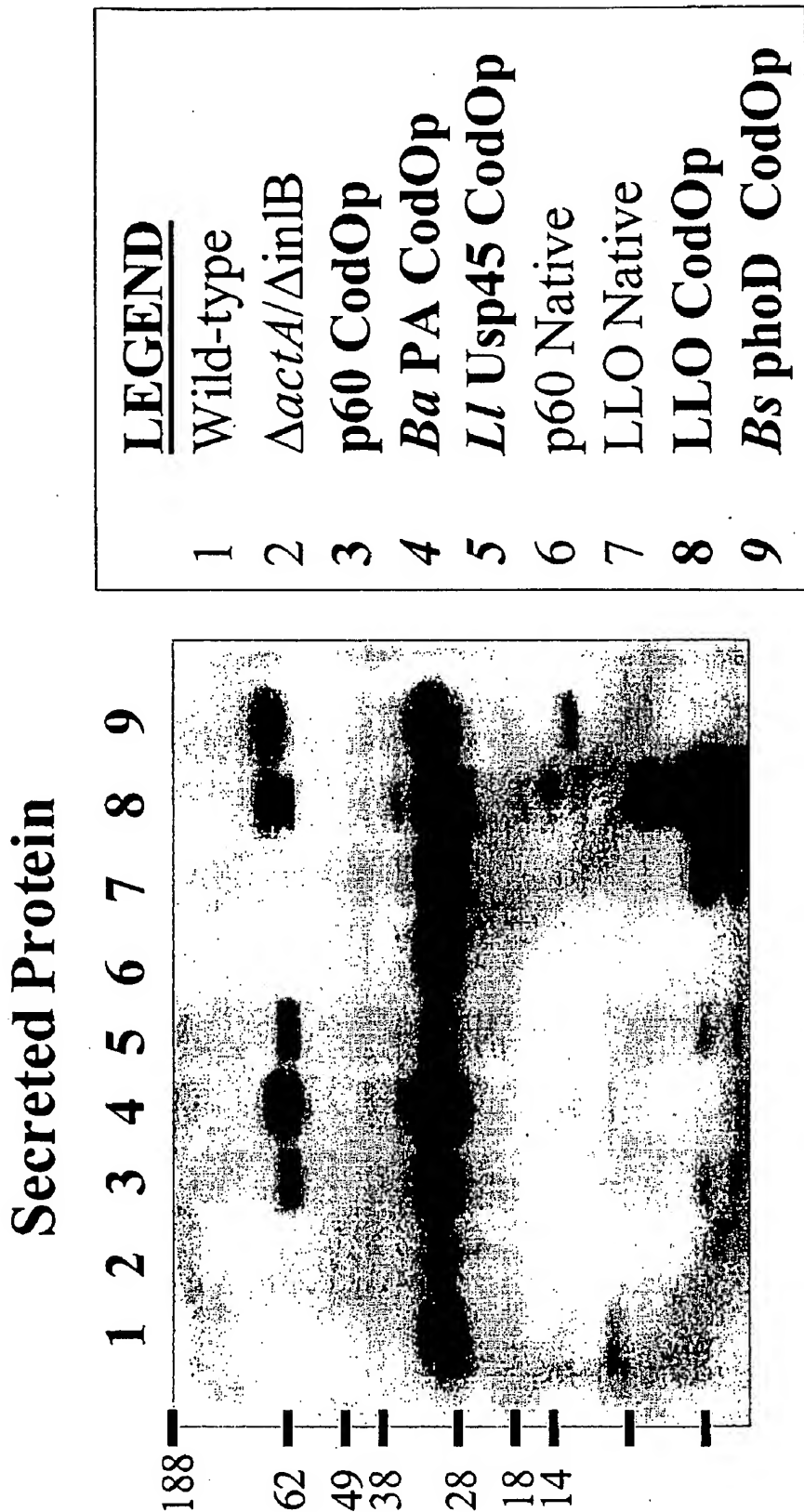


FIGURE 48B

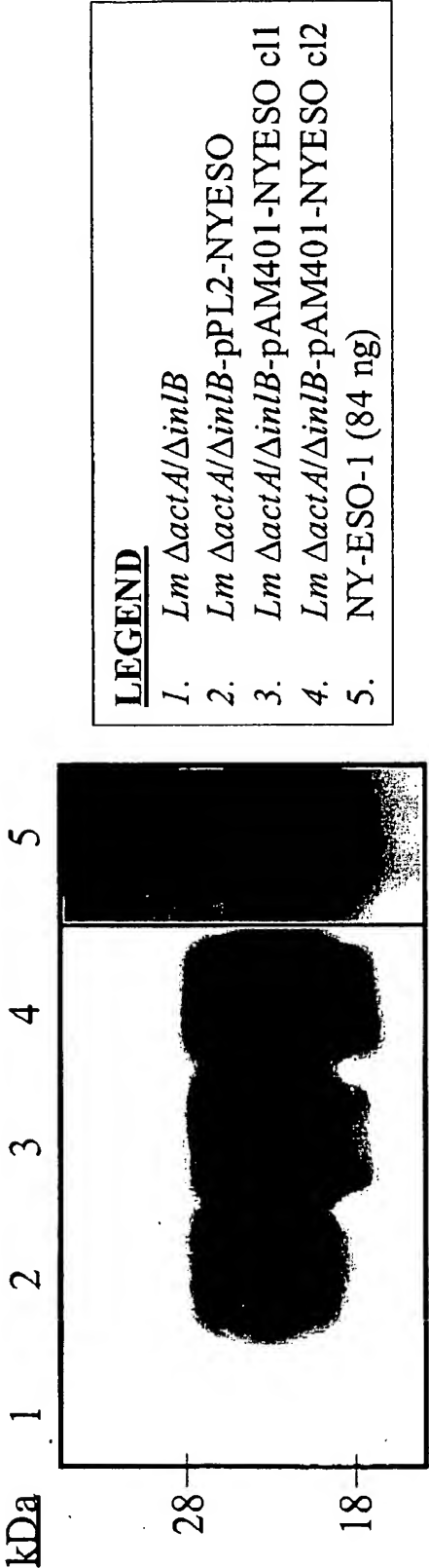


FIGURE 48C

Coding sequences of phEphA2KD:

GGATCCCAAGGAAAGAGTCGTACTTTAGATTTCGCAGCAGCAGGAGGAGATTAGGTAAGTAACTCATCCATATGGC
 AAGAGCTGGGATTTAATGCAAAACATATGCAACGATATGCCAATTTATATGTACTCCGTATGTAATGTAATGAGCGGTGATC
 AAGATAACTGTTACGTACTAATTGGGTTATCGAGGTGAAGCAGAAAGAAATTTTATTGAACCTTAAATTTACTGTTCTGTA
 CTGTAATAGTTTCCAGAGGGGCTCATGTAAAGAAACATTCATCTATATATGCGGAAAGCGATCTTGATATAGGT
 ACAAAATTTCCAAAACGTTTATTACTAAAATTGATACAAATAGCTCCAGATGAATCACTGTAAGTTCGGAATTTGAAAGCTC
 GTCATGTAAAATTAATGTAGAAGACGCGAGTGTGGTCCACTAACTAGAAAAGGAATTTATCTGCTTCCCAAGATATAGG
 GGCTTGCAGCATTTGTTATCCGTTCGTTACATATAAAAATGTCCAGACTACTTCAAGGCTTAGCACAATTTCCAGAA
 ACAATTGCGGGCTCAGATGCGCCATCACTTGCAACTGTGGCGGTACATGTGTTGATCATGCTGTTGTGCCACCAAGGAGGAG
 AGGAACCTCGCATGCACTGTCAGTAGATGGTGAATGGTTAGTCTTATGGTCAATGTTTATGTCAAGCCGGTTATGAAAA
 AGTTGAAGATGCTTGCAAGCATGCTCCCAAGGTTTAAATTCGAAGCTAGTGAATCTCCATGCTTAGAATGTCCAGAA
 CACACATTAACCAAGTCCAGAAAGGTGCAACGTCTGTGAATGCGAAGAAAGGTTTTTTCGTGCCCCACAAGATCCAGCCTCAA
 TGCTTGTACACGACCGCTTCTGCCACACTATTACAGCCGTAGGAATGGCGCTAAAGTAGAGTTACGATGGACACC
 GCCTCAAGATAGTGGAGGCGGTGAAGATATGTTTATTCGGTTACTTGTGAACAATCTGCGCAGAAAGTGGTGAATGCGG
 GCCTTGCGAAGCATCAGTTAGATATTCGGAACCCACACACCGGTTAACTAGAACTAGTCTCACAGATCAGACTTAGAACCC
 ACACATGAATATACATTTACATTTAGTTGAGGCACTGTAATGGATATCTGGTTAGTTACATCAGCTCTTTCGCACAGCAATCG
 GTCTCTATTAAACCAACTGAACCGCCAAAAGTAAAGATTAGAAAGGGGCTTCGACAAACATCACTTCCGTAAGTTGGTCAATTC
 CACCACCAACAATCAGCGTTTGGAAATATGAAGTTACATACAGAAAAGGAGATTTCGAATAGTTATATGTTAGAC
 GTACAGAAGGATTCAGCGTAACCTAGATATTAGCTCCAGATACAACATATTTAGTAGAGGTGCAAGCAATTAACACAAG
 AAGGACAAAGGGGGGCTCACGAGTTTCATGAATTTCAACAATTAAGAAAGAAAGAAATCAAGAGCAGTCATCAATCA
 CCAGAAGATGTTTATTTTCAAGTCTGAACAGTTGAACCACTTGAACACCTATGTTGATCCACACACATACGAAAGACCCAA
 ACCAAGCGGTCTTAAATTACAAACCGAAATTCATCCATCATGCGTAACTCGTCAAAAAGTGTGCGAGCTGGAGAAATTCGG
 GGAAGGTATACAAAGGCATGTTGAAAACCTCAAGTGTGTAATAAAGAAAGTCTCTGTAGCAATTTATGACTTTAAAGCAGGTA
 TACAGAAAACACGAGTTGATTTTTAGCGGAAGCTGGTATCATGGGACAAATTCGCAATCAATAATAATAGACTTGAA
 GGTGTTATCTCTAAATATAAACCAATGATGATTAATTAATGTAATATGGAACAAAGGTGCTTATAGATAAATTTCTACGCGAAA
 AAGATGGTGAATTTCTGCTCTCAATTAGTTGGTATGTTACGTGGCATCGCTCAGGTATGAATATCTTGCCAAACATGAAT
 TATGTACATAGATTAGCGGCTCGAAATATCTGTAAATTCCAAATTAGTGTGCAAGTTAGTATTCGGTTTAAAGTCG
 AGTATTAGAAAGATGATCCAGAACCACTACTACTCTCGGGGGTAAATTCGGATCCGTTGGACAGCACCGGAAAGCAAT
 TTCATATCGTAAATTACATCTGCAAGCGATGTTGGAGTTTCGGAAATGTGATGTGGAAAGTAATGACATACGCGGAACCGT
 CCATATTGGGAAATGTCAACCAACCATGAAGTAAATGAAGCGAATTAACGATGTTTTCAGATTAACCAACCCCAATGGACTGTCCAT
 CAGCAATTTTCAACTAATGATGCAATGCTGGCAACAAGAAAGAGCTAGAACCTTAAATTTGACAGACATTTGTTTCAATTTT
 AGACAAACTAATTCGTGCGCCAGATGCTTAAACCTAGCTGATTCGATCCACGGTATCAATTCGTCTCCATCAACA
 TCGGATCTGAAAGGTGTTCTTTTAGAACAGTAAAGCGAGTGGTTAGAAATCGATTAAATGCAACAGTATACAGAACATTTTA
 TGGCAGCCGGATACACAGCAATTGAAAAGTTGTGCAAAATGACAAATGATGATATTAAACGTAATGGAGTGGCTACCTG
 GCCACCAAAAACGTAATGCTTACTCCCTTTTAGGTTTAAAGACCAAGTAAATACAGTCGGAATTCCAATATGAGAGCTC

FIGURE 49

Mlu I sub-fragment of codon-optimized human EphA2 containing the actA-plcB intergenic region:

ACGCGTTTGGAATAATGAAGTTACATACAGAAATAAGGAGATTTCGAATAGTTATATGTTAGAC
GTACAGAAAGGATTCAGCGTAACCTAGATGATTTAGCTCCAGATACAAATATTTAGTACAGGTGC
AAGCATTAACACAAGAGGACAAGGGCGGCTCAGAGTTCATGAATTTCAACATTATAAAA
CACAGAAACGAAAGAAAGTGAGGTGAATGATGGCATATGATAGTCGTTTGTGATGAATGGGT
TCAAAATTAAGAAAGAAAGTTTCAAAATAATACATTTGATCGTCGTAATTTATTCAGGTGC
AGGTAAATTGCAAGTTTAAAGTTTAAAGTTTAAAGTTTAAAGTTTAAAGTTTAAAGTTTAAAG
AAGAAAGAAATCAAGAGCAGTCAATCACCAGAAAGATGTTTATTTTCAAAAGTCTGAACAGTTGAA
ACCATTGAAACCTATGTTGATCCACACATACGAAGACCCAAACCAAGCGGTCTTAAATTTAC
AACCGAAATTCATCCATCATGCGTAACCTCAAGTGGTAAAGAAAGTTCCTGTAGCAATTTAGACTCTTA
TATACAAAGGCATGTTGAAACCTCAAGTGGTAAAGAAAGTTCCTGTAGCAATTTAGACTCTTA
AAGCAGGGTATACAGAAACCAACGAGTTGATTTTTAGCGCAAGCTGATCATGGGACAAATTTT
CGCATCATAATAATTAGACTTGAAGGTGTTATCTCTAAATAATAACCAATGATGATTATTAATGA
ATATATGGAAACGGTGCTTTAGATAAATTTCTACGCGAAATAAGATGGTGAATTTCTGTCTTCA
ATTAGTTGGTATGTTACGTGGCATCGCTGAGTGAATATCTTGCCAAACATGAATTTATGTACAT
AGAGATTTAGCGGCTCGAAATAATCTTGTAAATTCCTAAATTTAGTGTGCAAGTTAGTGAATTCGGTT
TAAGTCGAGTATTAGAAAGATGATCCAGAAACCTATACCTTCTCGGGGGTAAATTCGGATCC
GTTGGACAGCACCGGAAGCAATTTTCATATCGTAAATTTACATCTGCAAGCGATGTTGGAGTTTCG
GAATTGTGATGTGGGAAGTAAATGACATACGGCGAACGTCCATATTGGGAATTTGCAACCATGAA
GTAATGAAAGCGATTAAACGATGGTTTCAGATTACCAACCCCAATGGACTGTCCATCAGCAATTTAT
CAACTAATGATGCAATGCTGGCAACAAGAAAGAGCTAGAAAGACCTAAATTTGCAGACATTTTCA
ATTTAGACAAACTAATTCGTGCGCCAGATAGTCTTAAACCCCTAGCTGATTTCGATCCACGCGT

FIGURE 50

hly promoter-70 N-terminal p60 amino acids:

GGTACCTCCTTTGATTAGTATATTCCCTATCTTAAAGTTACTTTT
ATGTGGAGGCATTAAACATTTGTTAATGACGTCAAAAGGATAG
CAAGACTAGAAATAAAGCTATAAAGCAAGCATATAATATTGCG
TTTCATCTTTAGAAGCGAATTTTCGCCAATATTATAATTATCAA
AAGAGAGGGGTGGCAACGGTATTTGGCATTATTAGGTTAAA
AAATGTAGAAAGGAGAGTGAAACCCATGAATATGAAAAAAGC
TACGATTGCAGCTACAGCCGGCATTTGCCGTAAACAGCTTTTGCA
GCACCAACTATTGCCCTCAGCCTCTACAGTTGTGTGTCGAAGCAG
GAGACACATTATGGGGAATCGCACAAATCAAAAGGTACAACG
GTTGATGCTATTAAAAAAGCGAATAATTTAACAACAGATAAA
ATCGTGCCAGGTCAAAAAACTGCAG

FIGURE 51

KpnI-*BamHI* sub-fragment of pPL2-hlyP-Np60 CodOp(1-77):

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTAAACATTTG
TTAATGACGTCAAAAGGATAGCAAGACTAGAAATAAAGCTATAAAGCAAGCATATAATATTGC
GTTTCATCTTTAGAAAGCGAATTCGCCAATATTATAATTATCAAAAGAGAGGGTGGCAAAACG
GTATTTGGCATTATTAGGTTAAAAAATGTAGAGGAGAGTGAACCCCATGAATATGAAAAAAA
GCTACGATTGCAGCTACAGCCGGCATTGCCTAACAGCTTTTGCAACCACTATTGCCCTCA
GCCCTACAGTTGTGCGAAGCAGGAGACACATTATGGGAATCGCACAATCAAAAGGTACA
ACGGTTGATGCTATTAAAAAAGCGAATAATTAAACAACAGATAAAATCGTGCCAGGTCAAAA
ACTGCAGGTAAATAATGAGGTGCTGCTGCTGAAAAAACAGAGAAATCTGTAGCGCAACTTG
GTTAAACGTCCGTA CTGGCGTGGTGTGATAACAGTATTATTACGTCCATCAAAAGGTGGAAC
AAAAGTAACTGTTGAAACAAACCGAATCTAACGGCTGGCACAAAATTACTTACAACGATGGAA
AAACTGGTTTCGTTAACGGTAAATACTTAACAGCAAGCAGTAAGCACTCCAGTTGCACCAA
CACAAAGAGTGA AAAAGAACTACTACTCAACAGCTGCACCTGTTGCAGAAACAAAACCT
GAAGTAAACAACTACACAAAGCAACTACACCTGCGCTAAAGTAGCAGAAACGAAAGAAAC
TCCAGTAATAGATCAAAATGCTACTACACGCTGTCAAAAGCGGTGACACTATTTGGGCTTT
ATCCGTAAATAACGGTGTCTGTTCAAGACATTATGTCATGGAATAATTTATCTTCTTCT
ATTTATGTAGGTCAAAAGCTTGCTATTAAACAACCTGCTAACACAGCTACTCCAAAAGCAGAA
GTGAAAACGGAAGCTCCAGCAGCTGAAAACAAAGCAGCTCCAGTAGTTAAAGAAAATACTAA
CACAAATACTGCTACTACAGAGAAAAGAAACAGCAACGCAACACAAACAGCACCTAAAG
CACCAACAGAGCTGCAAAACCCAGCTCCTGCACCATCTACAAACACAATGCTAATAAAACG
AATACAAATACAAATACAAACAATACTAATACACCATCTAAAAATACTAATACAACTCAAAT
ACTAATACGAATACAACTCAAATACGAATGCTAATCAAGTTCTTCCAAACAATAACAGCAAT
TCAAGTGCAAGTGCTATTATTGCTGAAGCTCAAAAACACCTTGGAAAAGCTTATTCATGGGGT
GGTAACGGACCACTACATTGATTGCTCTGGTTACACTAAATATGTTTGTAAAGCGGGT
ATCTCCCTTCCACGTACATCTGGCGCACAATATGCTAGCACTACAAGAAATTTCTGAATCTCAAG
CAAAACCTGGTGAATTAGTATCTTCGACTATGTTAGCGGAAATTTCTCACATTTGTTATGT
TGGTAATGGTCAAATGATTAAACGCGCAAGACAATGGCGTTAAATACGATAACATCCACGGCTC
TGGCTGGGGTAAATATCTAGTTGGCTTCGGTCGGTATAATAAGGATCC

FIGURE 52

KpnI-*BamHI* sub-fragment of plasmid pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin:

GGTACCTCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCATTAAACATTTGTTAATGACGTCAAAAGGATAGCAAGACT
 AGAATAAGCTATAAAGCAAGCATATAATATTGCGTTTCATCTTTAGAAAGCAATTCGCCAATATTAATAATTCAAAAGAGAGGGGTGGC
 AAACGGTATTGGCATTTAGGTTAAATAATGTAGAGGAGTGAACCCATGAATATGAATAAAGCTACGATTGACGCTACAGCCGGC
 ATTGCCGTAAACAGCTTTGACGACCAACTATTGCTCAGCTCTACAGTTGTTCGAAAGCAGGAGACACATTAATGGGAATCGCACAAATCA
 AAAGTCAAAACGGTTGCTATTAAATAAGGAATAATTAAACAACAGATAAATGCGCCAGGTCAAAAACCTGACGGCAATTGCCAACTG
 CACGTCCATTACTAGGTAGTTGCGGTACACCAGCACTAGGTTCTATTATTTTGTATTCTCTAGTTGGTTCAACCAAGTCGTACATT
 AGCAGGTAAACAGGTCAAGGAGCAGCCACTGACGGTGTTAATACGAAATCCACAAATATATCAAGTTTAAAGTCCACGTCATTAATTAG
 GTTTCCATGTGCAGAAATTTCAGGTTTAAAGTACAGAAACGTGTCGTGAGTTAGCAGTTGCAATTAGCACAATAAAGTTAAATTATCTACAG
 AACAGTTACGTTGTTAGCCCATAGATTAAAGCGAAACACAGAAAGACTAGATGCATTCCTTAGACCTCTTTTATCTTAAATCCAGATG
 CATTTTCAGGACCAACAGCATGACAGTTTATAGTCGAATTACAAAGCCCAATGTTGATTATACCTCGTGGGCTCCTGAAAGACAAC
 GTTATTACCTGCTGCATTAGCATGCTGGGTTCGCGGTAGCTTAAAGTGAAGCCGATGTCGTGCTTAGGGGTTAGCATGIGATT
 ACCTGGTCTTTGTCAGAAATCAGCAGAAAGTTTATACCGAGATTAGTTTCATGCCAGACCTTTAGATCAAGATCAACAAGAGGCGAG
 CTAGAGCAGCTCTTCAAGGAGGAGGCCCACTATGGCCCAACCAAGTACATGGAGTGTCTTACAATGGATGCGTTAAGAGGTTTATACCG
 GTTTTAGGACAACCAATTATTGCTAGTATTCCACAAGGCAATGTAGCAGCATGGCGTCAACGTAGTTCTGTCGTGTCGTCGCGGACAAACA
 GAACGTACAATTCTACGTCCAAGATTTCGTAGAGAAAGTAGAAAACCGCGTGTCTAGTGGCAAAAAGCACGTGAAAATTGATGAAAGTT
 TAATTTTTTATAAAAATGGAAATTAGAAAGCATGTGTGATGCGAGCATTAAGCTACACAATGGATCGTGTAAATGCTATTCCATTCCACAT
 ATGAACAATTAGATGTTTAAAGCATAAATTAGACGAATTATATCCACAAGGTTATCCAGAATCAGTTATTCACAACATTTAGGTTACTTATTT
 TAAAAATGAGTCCAGAAAGACATACGAAATGAATGTAATGTAAGAAACATTAAAGCGCTTTTAGAAGTTAACAAAGGTCATGAAAT
 GAGTCCACAAGTTGCTACGTTAATTGATAGATTGCTTAAAGCGCTGTCCTCAATAGATAAAGATAGTATGATACATTAACACAGCATTTTATCC
 TGCTACTTATGCACTTATCACAGAAATTAAGTTCGTTCCACCGAGTAGTATCTGGCAGTTCGTCGCGCAAGATTTAGATACATGCGA
 CCCACGTCAATTAGATGTTTATCCAAAAGCAAGATTAGCTTTCCAAAATATGAACGGTAGTGAATATTCGTAAATAATTCATTCCTTTT
 AGTGGTGCACCAACTGAAGATCTAAAGCATTAAGCCAAACAATGTAAGTATGGATTAGCTACGTTTATGAAATTAACGTACAGATGCAG
 TTCTACCAATTACAGTTGCAGAAATTCAAAATTAATTAGTCCACACGTAGAAAGGATTAAAGCAGAAAGAACGTCAACGTCAGTTCGCGAT
 TGGATTTTACGTCAACGTCAAGATGATTAGATACATTAAGTTTACAGGCGGTATTCGGAATGGATATTTAGTGTAGATTATCT
 GTTCAAGAAAGCATTAAGTGGTACCGTGTTTATTAGTCCAGGTCAGTTTAAACAGTGTAGCAATTATTAATAGCCAGTACATTAGCTCTG
 CAGGTAATAATGAGGTGCTGCTGTAATAAACAAGCAAGAAATCTGTTAGCCAACTTGGTTAAACGTCCGTAATGCTGCTGGTGTGATGA
 CAGTATTATTACGTCCATCAAGGTGGAACAAAAGTAACGTGTGAACAACCGGAATCTAACGGTGGCACAAAATTACTTACAACGATGGAA
 AAACGTGTTTCGTTAACGGTAAATCTTAACGTGACAAAGCAGTAAAGCACTCACAGCAACTACACCTGCGCTAAAGTACGAGAAACGAA
 TCAACAAGCTGCACCTGTGCAGAAACAAAACCTGAAGTAAACAACAACTACAGCAACTACACCTGCGCTAAAGTACGAGAAACGAA
 GAAACTCCAGTAATAGATCAAAATGCTACTACACACGTGTCAAAAGCGGTGACACTATTTGGCTTTATCCGTAAAATACGGTGTCTGTT
 CAAGACATTATGTCATGGAATAATTTATCTTCTCTTATTTAGTGGTCAAAAGCTTGTCTATTAAACAACTGCTAACACAGCTACTCCA
 AAAGCAGAAAGTGAACCGAAGCTCCAGCAGCTGAATAAACCAAGCAGCTCCAGTAGTTAAAGAAATACTAAACAATACTGCTACTACAG
 AGAAAAGAAACAGCAACGCAACAAACAACAGCACCTAAAGCCCAACAGAAAGCTGCAAAAACCACTCTGCACTCTCAAAACACAA
 ATGCTAATAAAGCAATACAAATACAAATACAAATACATATACACCATCTAAAATACTAATACAAACTCAATACTACGAATAC
 AAATCAAAATACGAATGCTAATCAAGGTCTTCCAAACAATAACAGCAATTCAGGTGCAAGTCTAATTTGCTGAAGCTCAAAAACACCTTG
 GAAAGCTTATTCATGGGTGGTAAACGACCAACTACTGTTGCTGTGCTGTTACACTAATAATGTTATTTGCTAAAGCGGGTATCTCCCTTC
 CACGTACATCTGGCGCAATATGCTAGCACTACAGAAATTTCTGAATCTCAAGCAAAACCTGGTGAATTAGTATTTCTCGACTATGGTAGCG
 GAATTTCTCACATTGGTATTATTTGTAATGGTCAATGATTAAACGCGCAAGACAAATGGCGTTAAAATACGATAACATCCACGGCTCTGGCT
 GGGGTAATAATCTAGTTGGCTTCGGTCCGTATATAAAGGATCC

FIGURE 53

KpnI-*Bam**HI* sub-fragment of plasmid pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin Δ SP/ Δ GPI:

GGGTACCTCCTTTGATTAGTATATTCCTAATCTTAAGTTACTTTTATGTGGAGCAATTAAACAATTTGTTAATGACGTCAAAAAGGATAGCAAG
ACTAGAAATAAGCTATAAAGCAAGCATATAATATTGGTTTCATCTTTAGAAGCGAATTTGCGCAATATATATAATTATCAAAAAGAGAGG
GGGCGCAAAACGGTATTGGCAATTTTAGTTGCAAAAATGTAAGAGGAGAGTGAACCCATGAATATGANAAGAAAGCTACGATTGCAAGT
ACAGCGCATTCGGTTAACCAGCTTTGACAGCAACTATGCGCTCAGCTCAGTTGTTGCGAAGCAGGAGACACATTAGCGGG
AAATTCGCACAAATCAAAAGGTACAAACGGTTGATGCTATTAAAAAGAGCAATAATTTAACACAGATAAAAATCGTGCCAGGTCAAAACAACTG
CAGCGTCAATATTAGGTTTCCATGTGCGAAGATTTACAGTTTAAATGACAGAGTGTCCGTGAGTTACGAGTTGCAATTAGCACAAA
ACGTTAAATATTACAGAAACAGTTAGTTTACGCCATAGATTAAGCGAACCCAGCAAGCTTAGATGCATTCCTTTAGACCT
TCTTTTATCTTAAATCCAGATGCAATTTACGGACCACAAGCATGTACACGTTTTTTTAGTCGAATTAACAAAAGCCAATGTTGATTTAT
TTCTGTGGGTCCTGAAACACAGTTTATACCTGCTGATAGCATGTCGGGTGTTCCGGTGTTATTAACGAGATTAGTTGAAGCCGAT
TTCTGTGCTTAAAGGGGTTAGCATGTGATTACCTGTGCTTTCGTTGCGAATCAGCAAGATGCTGTTATTAACGAGATTAGTTTCATGCTC
TTAGATCAAGATCAACAAAGAGGCGCTAGACAGCTCTCAAGGAGGCGCCACCATATGCCCAACCAAGTACATGGAG
TGTGTTCTACAATGGATCGTTAAGAGTTTATACCGGTTTTAGGCAACCAATTTATTCGTATTCACAAAGCAATGTAGCAGCATG
GCTGCTAGTTCTCGTATCGCTTGGCGACCAACAGCAAGCTACAAATCTACGTCGAAGTTTCGTAGAGAAAGTAGAAAAAC
GCGTGTCTAGTGGCAAAAAGCAGCTGAAATGATGAAGAACTTAAATTTTATAAAAATGGGAATTAGAAGCATGTGTCGATAGCAG
CATATTAGCTACACAAATGGATCGTTGATATGCTATTCATATCACATAGAACATTAGATGTTTTAAAGCATAAATTAGACGAATTA
TATCCACAAGTTATCCGAATCAGTTATTCAACATATTAGTTACTATTTTTAAAAATGATGCCAGAAGACATACGCAAAATGGAATGT
TACAAAGTTAGAAACATTAACAGCGCTTTTAGAAGTTTACAAAGGTCATGAAATGAGTCCACAGTTTGCTACGTTAATGATAGATTCG
TTAAGCGCGTGTCATATTAGATAAGATATCTAGATACATAACGACATTTATCTGGTCACTTATGCGTTATCACCCAGAAAGAA
TTTTAAGTCCGTTCCACCGAGTAGTATCTGGCAGTTCTGCGCAAGATTTAGATACATCGCACCCACGTCAATTAGATGTTTTATATCCA
AAAAAGCAAGATTGCTTCCAAAATATGAACGGTAGTGAAATTTCTGTAAAAATTCAAATCTTTTAGTGTGTCACCACTACGAAGATCT
AAAAAGCATTAAGCCAAACAAATGTAAAGTATGGAATTAGCTACGTTTATGAAATTTACGATACAGATCGAGTTCTACCATTAACGTCAG
AAAGTTCAAAATATTATAGGCTCACAGAGGATTAAAAAGCAAGAACCTCCAGTCCGATTCGCAATTTGATTTACGTCAAACG
TCAAGATGATTAGATACATTAGGTTTAGTTTACAAAGCCTGCAGGTAAATAATGAGGTTGCTGCTGTGTAATAACAGAGAAATCT
TTAGCGCAACTTGTTTAAAGCTGTACTGCGGTGTTGATACAGATTAATACGTTCCATCAAGAGGTGGAACAAAGTAGTAACTGT
TGAACAAACCGAATCTAACCGCTGGCACAAATTACTTCAACAGTAGGAATAAACTGTTTTCGTTAACGTTAAATCTTAACCTGACAAA
GCGATGAAGCTACAGTTGACCAACCAAGAAAGTGAAATAAGAAACTACTACTCAACAAAGCTGACCTGTTCCAGAAACAAAACCT
GAAATGAACAAACTACACAGCAACTACATCGCGCTAAAGTAGCAGAAACGAAAGAACTCCAGTAATAGATCAAAATAGCTACT
ACACAGCTGTCAAAGCGTGACATTTGGGCTTTATCCGTAAAAATACGGTGTCTGTTCAGACATATGATGATCGAATATATTT
ATCTCTCTCTTATTTAGTTAGTCAAAAAGTGTCTATTAAACAACTGCTAAACAGCATCTCCAAAAGCAGAGTGAACACGGAA
CTCCAGCAGCTGAAAACAAAGCAGCTCCAGTAGTTAAAGAAATACTACACAAATACTGCTACTACAGAGAAAAAGAAACAGCAA
CGCAACAAACAAACAGCACCTAAAGCAACCAAGAGCTGCAAAACCGAGCTCTGCACCATCTACAAACCAAAATGCTAATAAAGCA
ATACAAATACAAATACAAACAACTAATAACCACTAACTAATAACAACTCAAAATCTAATACGAATACAAACTCAAAATAC
GAATGCTAAATCAAGGTTCTTCCAACTAAATACAGCAATTCAAAGTGCAAGTCTATTATGCTGAAGCTCAAAACACCTTGGAAAGCTT
ATTATGGGTGGTAAACGGACCAACTACATTTGATTGCTGTGTTACACTAAATATGTAATTTGCTAAAGCGGGTATCTCCCTTCCACGTA
CATCTGGCGCAATATGCTAGCACTACAGAAATTTCTGAATACAGCAAAACCTGGTGATTATCTTCGACTATGTTAGCGGA
ATTTCTCACAATGTTATTTAGTTGGTAATGGTCAATATGATTAACCGGCAAGACAATGGCGTTAAATACGATAACATCCACGGCTCTGG
TTGGGTAAAAATATAGTTTGGCTTCGGTCCGCTTAATAAGGATCC

FIGURE 54

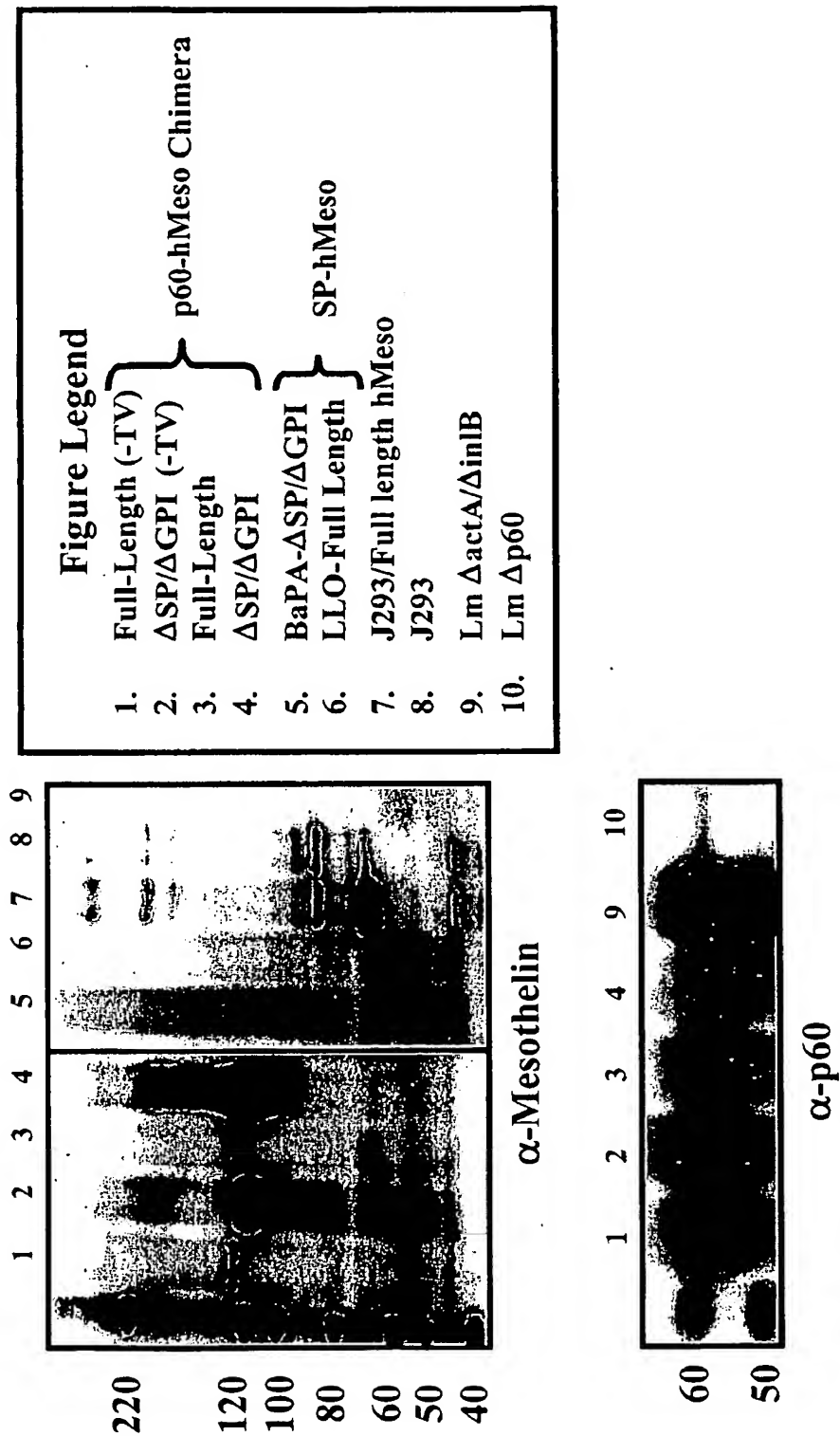


FIGURE 55

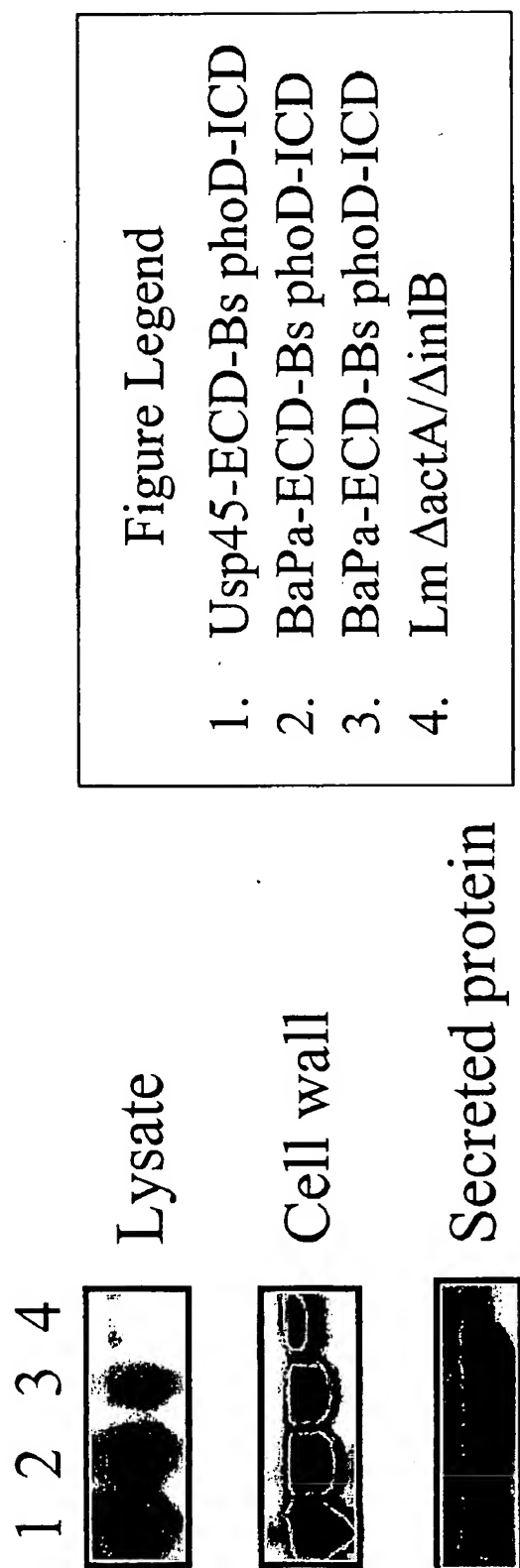


FIGURE 56

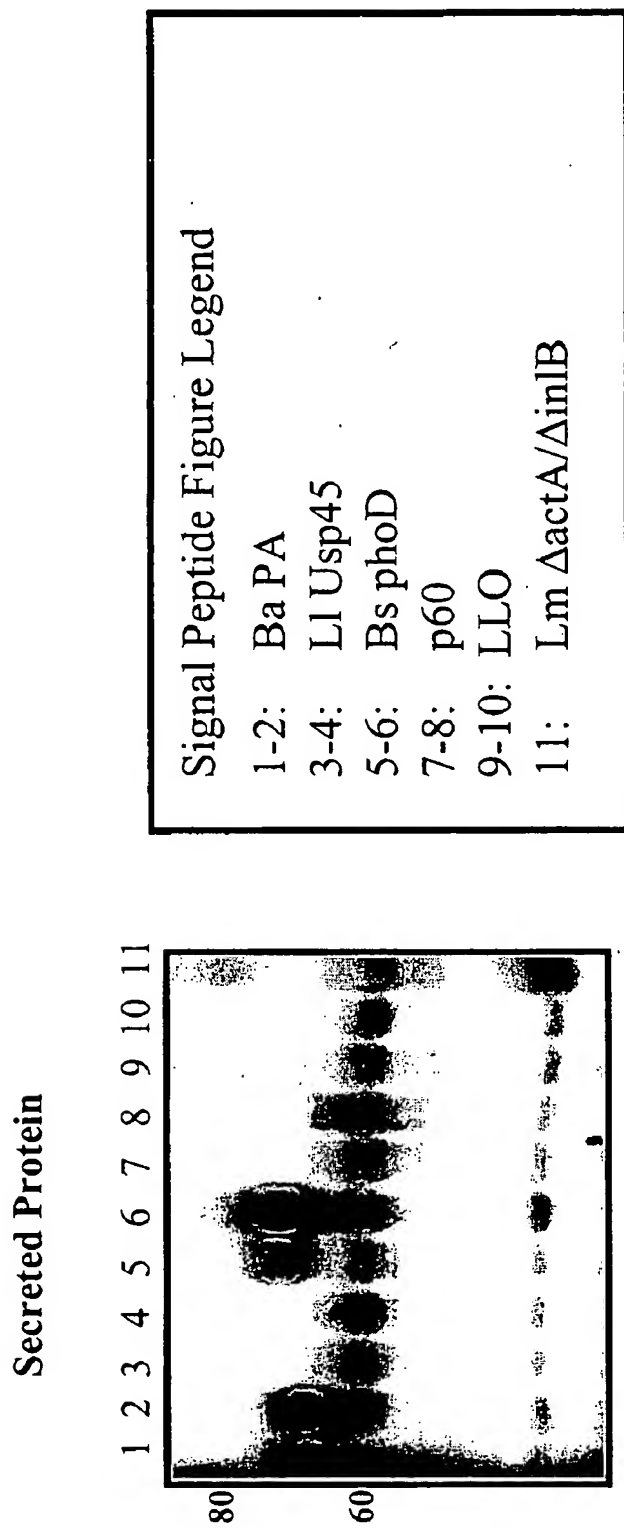


FIGURE 57A

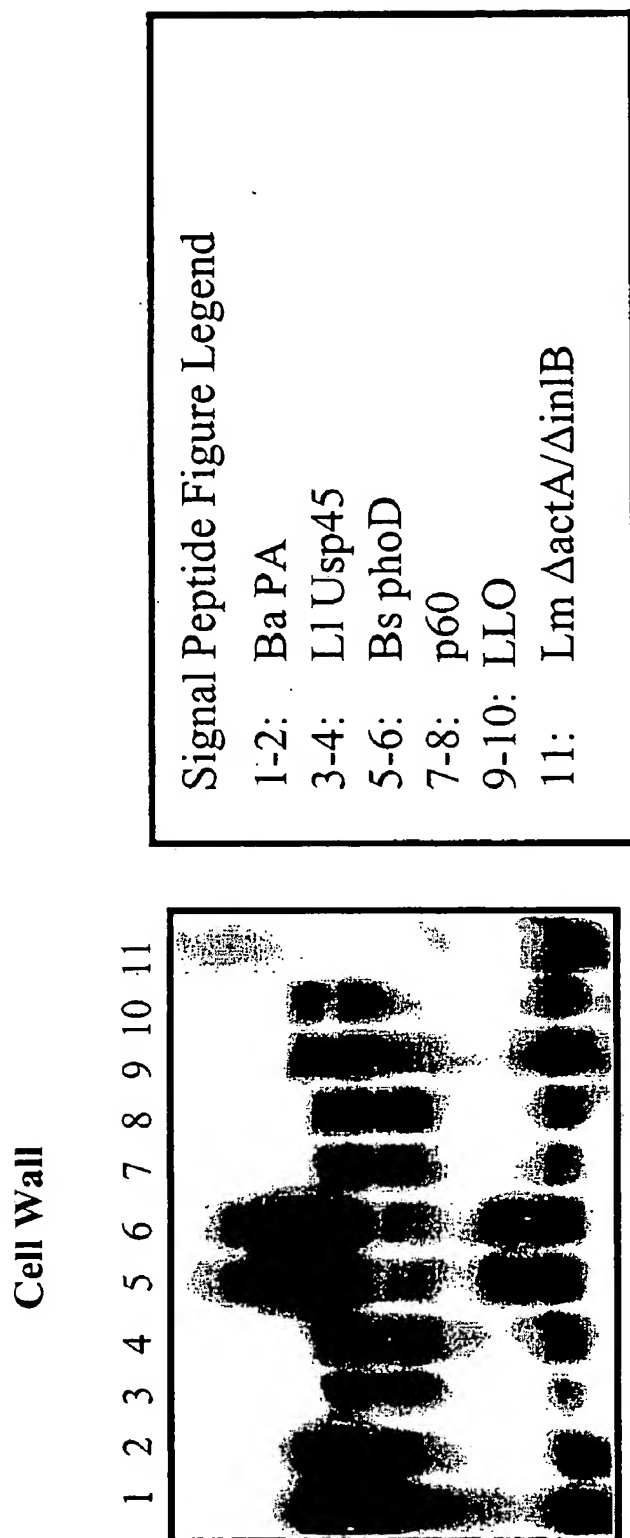
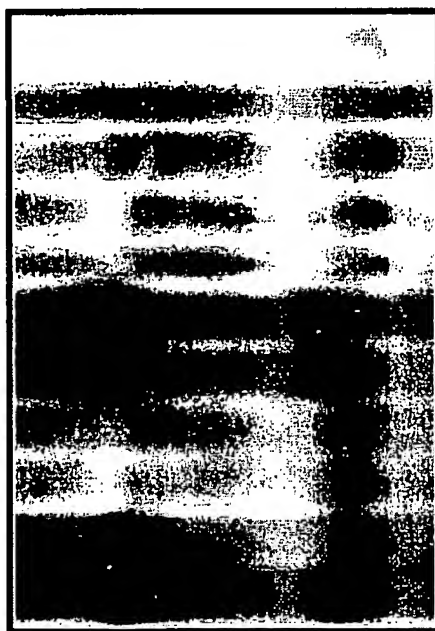


FIGURE 57B

Cell Lysate

1 2 3 4 5 6 7 8 9 10 11



Signal Peptide Figure Legend

- 1-2: Ba PA
- 3-4: Ll Usp45
- 5-6: Bs phoD
- 7-8: p60
- 9-10: LLO
- 11: Lm Δ actA/ Δ inlB

FIGURE 57C

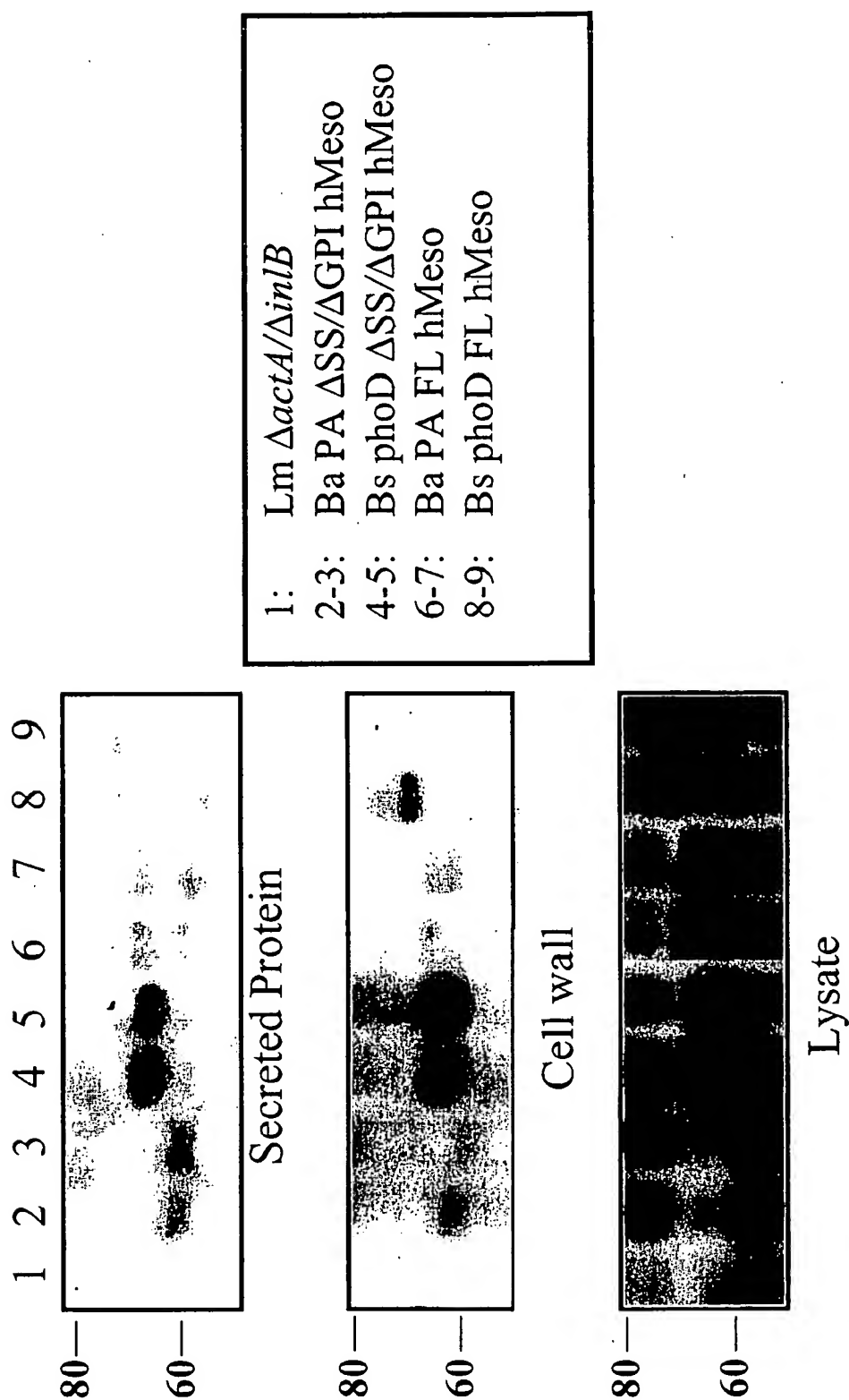


FIGURE 58

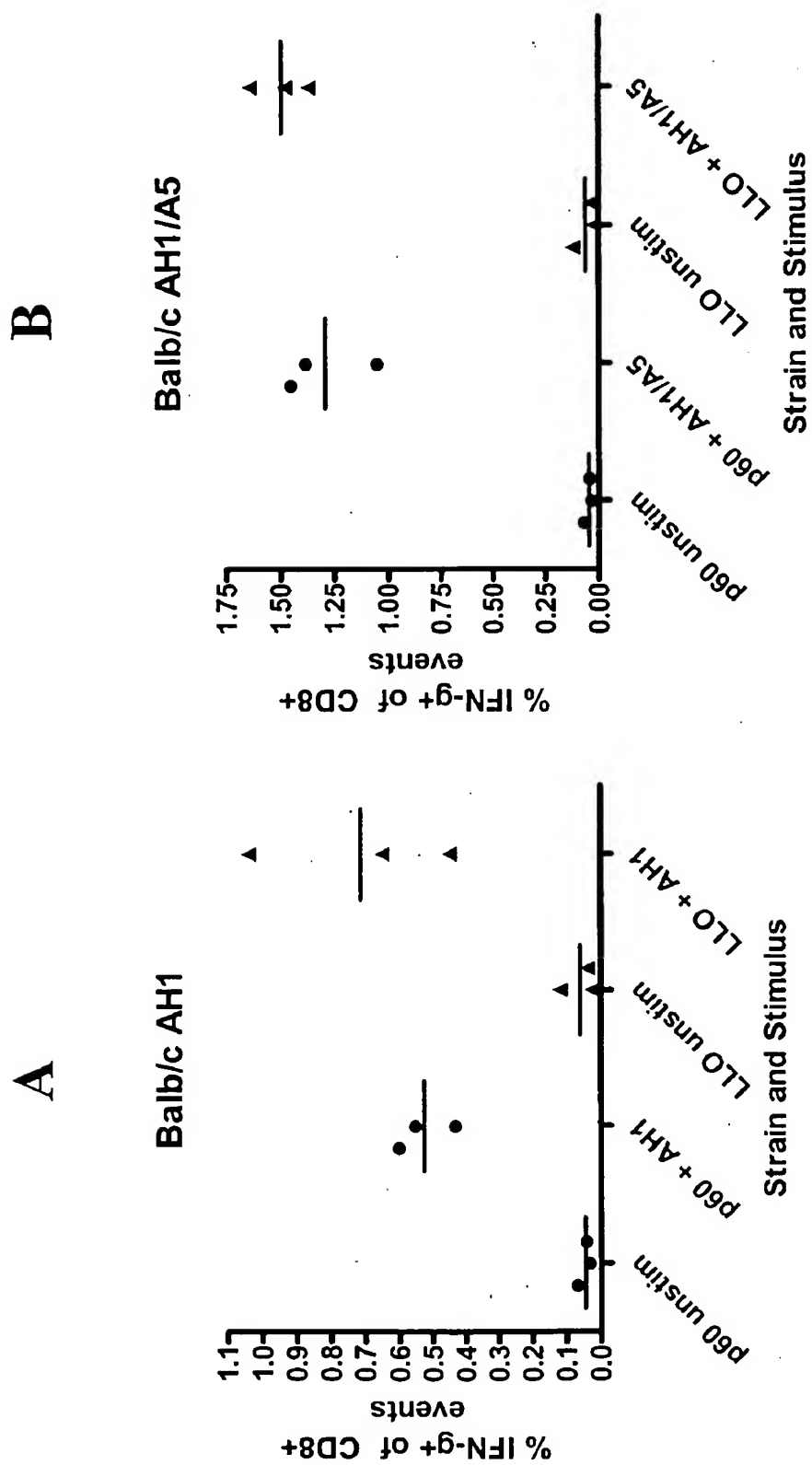


FIGURE 59

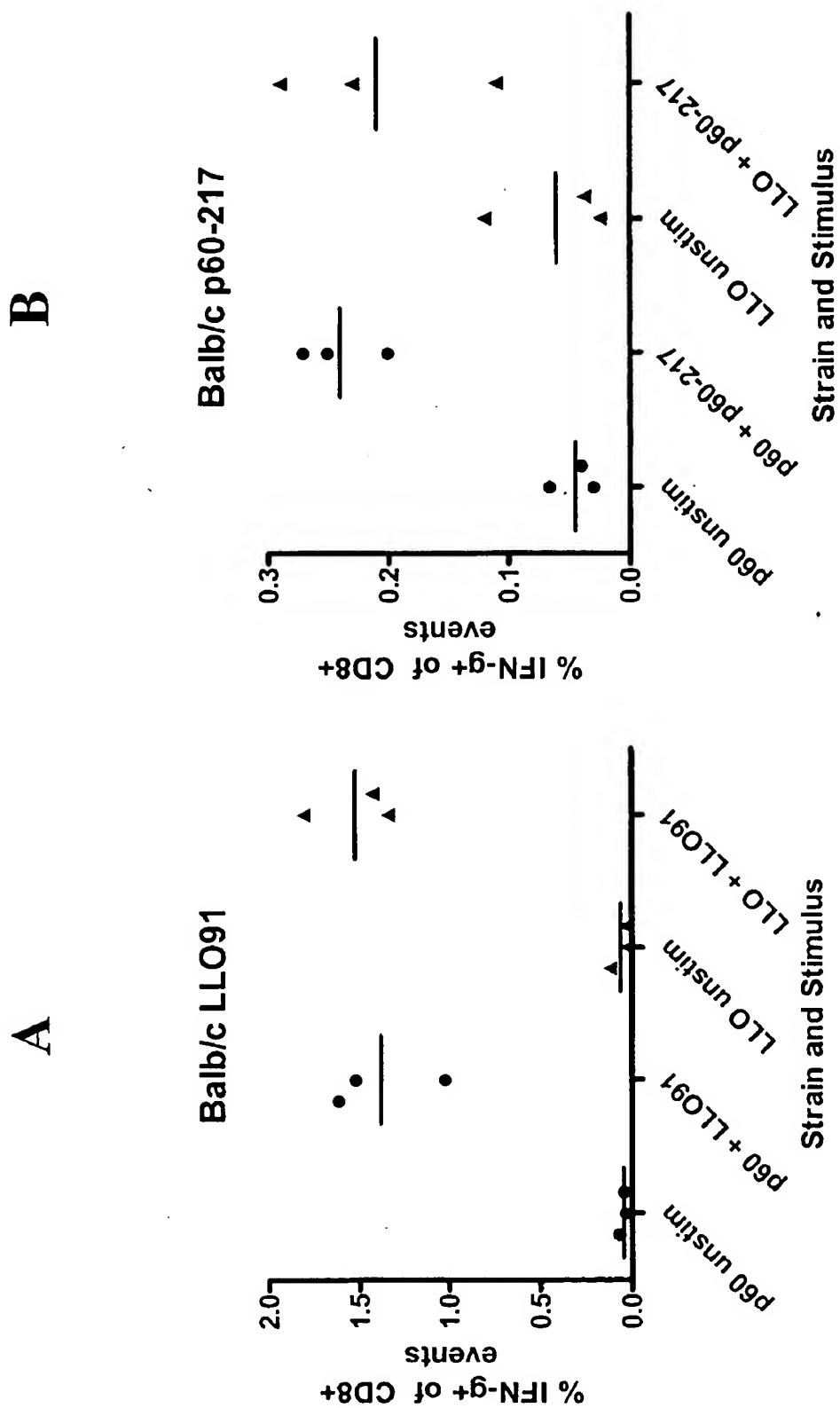


FIGURE 60

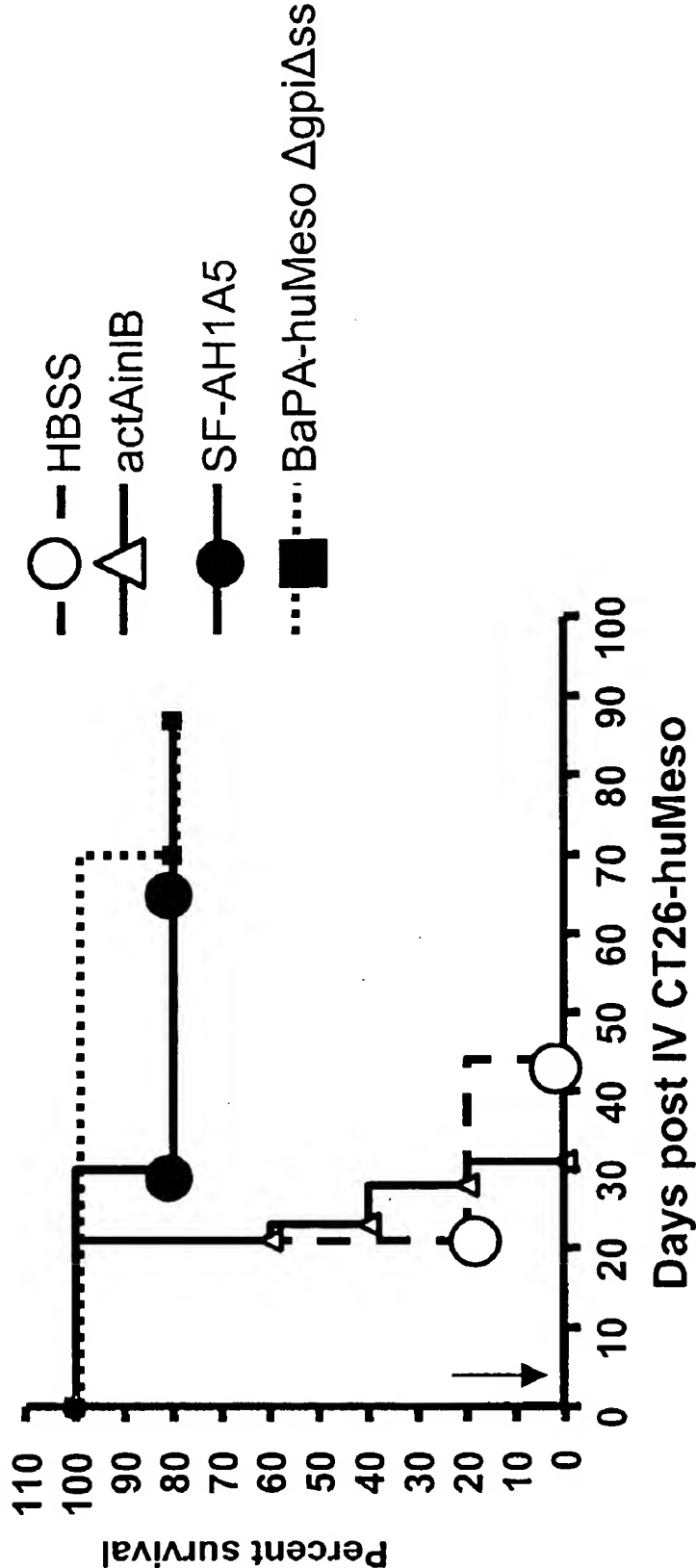


FIGURE 61

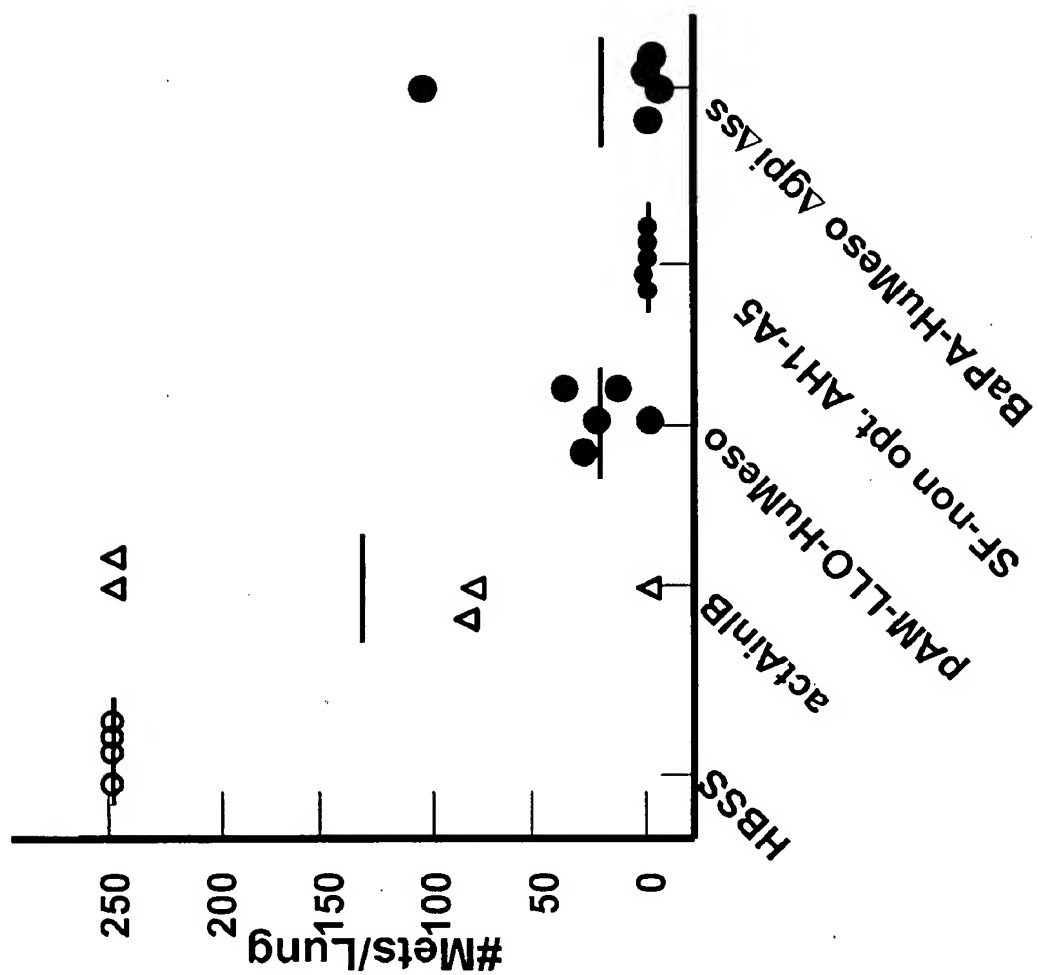


FIGURE 62

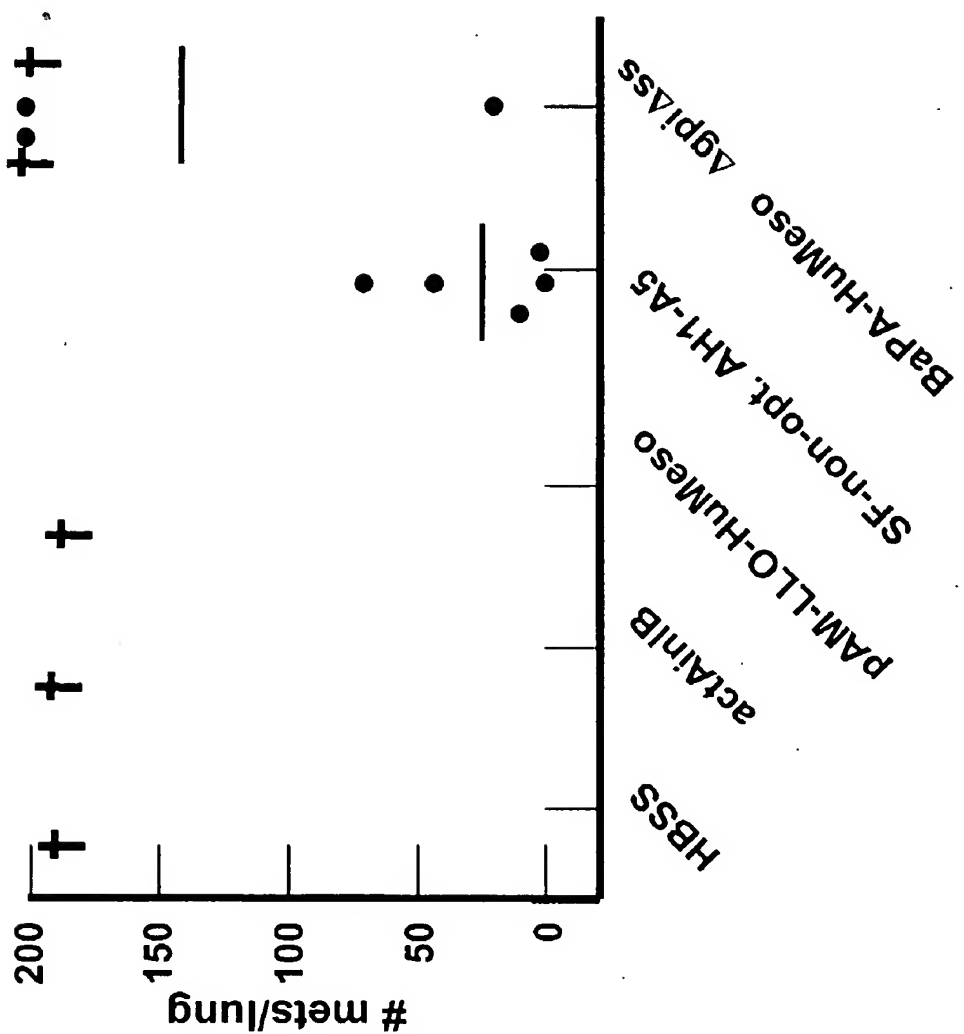


FIGURE 63

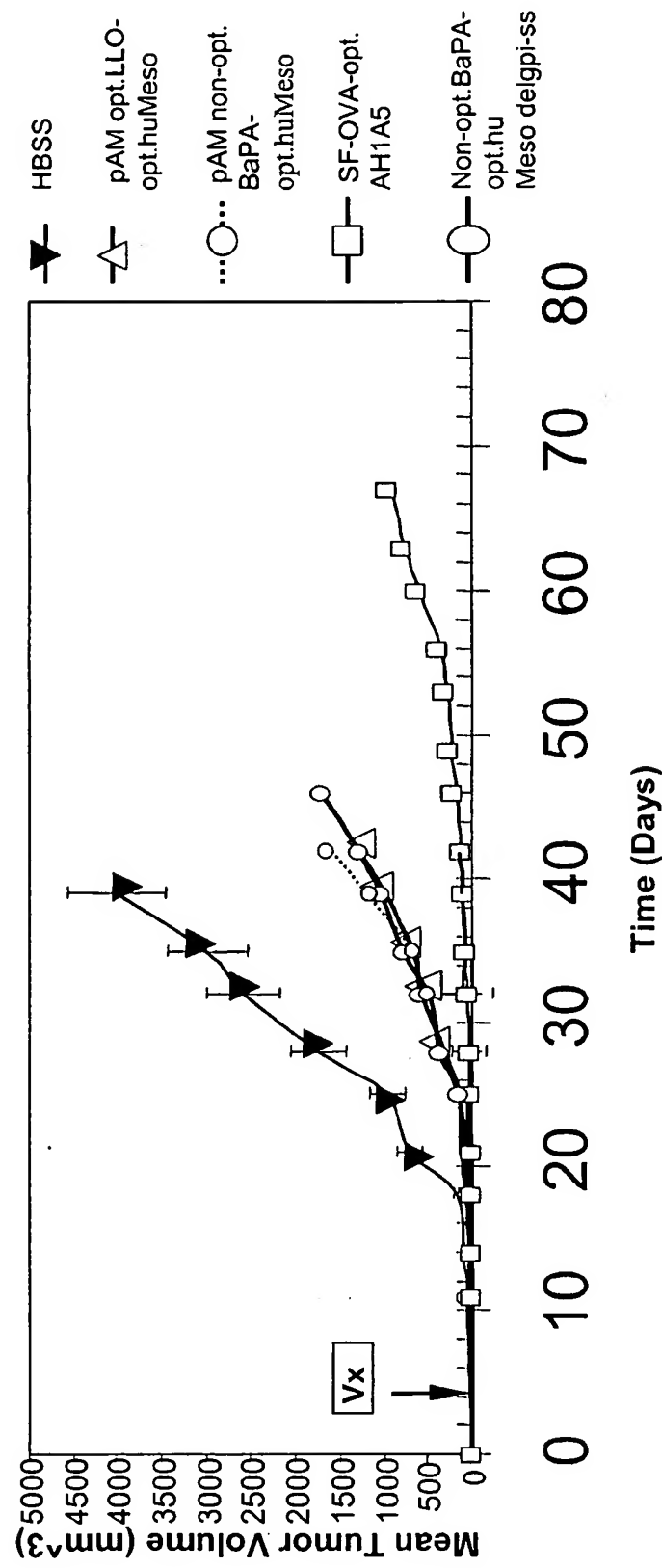


FIGURE 64

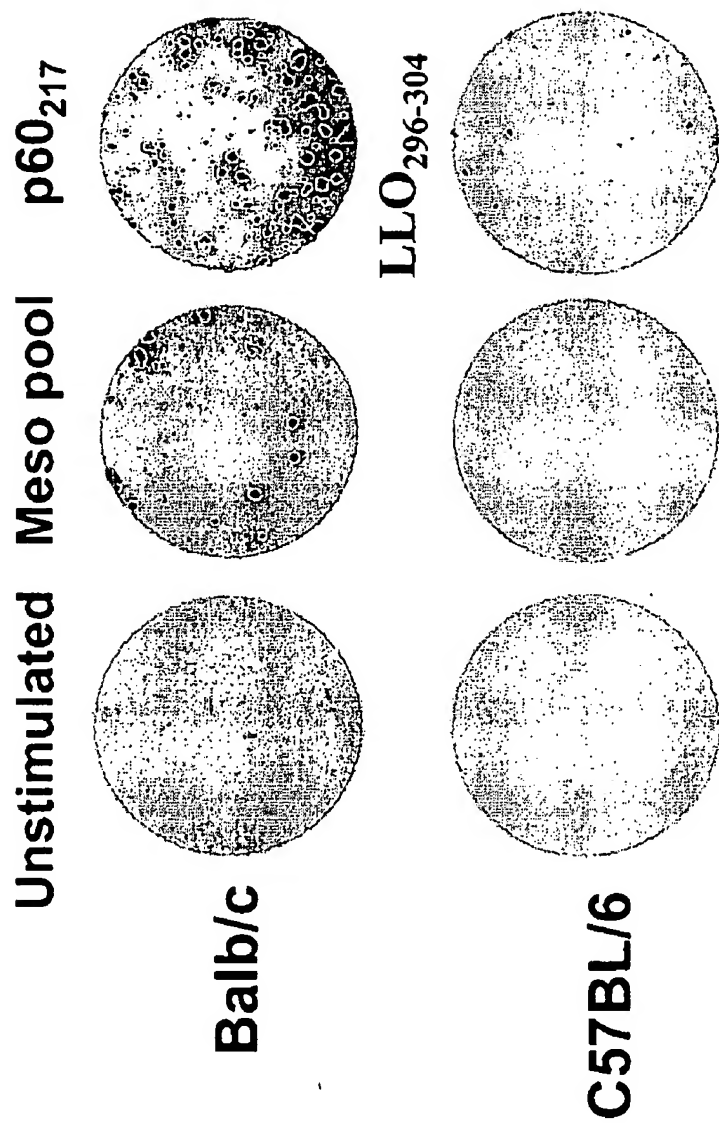


FIGURE 65

RECOMBINANT NUCLEIC ACID MOLECULES, EXPRESSION CASSETTES, AND BACTERIA, AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit under 35 U.S.C. §119(e) of each of the following U.S. provisional applications, the disclosures of each of which are hereby incorporated by reference in their entirety herein: U.S. provisional application Ser. No. 60/616,750, entitled "Bacterial Expression Cassettes, Bacterial Vaccine Compositions, and Methods of Use Thereof," by Thomas W. Dubensky, Jr. et al., filed Oct. 6, 2004 (Docket No. 282173003923); U.S. provisional application Ser. No. 60/615,287, entitled "Bacterial Expression Cassettes, Bacterial Vaccine Compositions, and Methods of Use Thereof," by Thomas W. Dubensky, Jr. et al., filed Oct. 1, 2004 (Docket No. 282173003922); U.S. provisional application Ser. No. 60/599,377, filed Aug. 5, 2004; U.S. provisional application Ser. No. 60/556,744, filed Mar. 26, 2004; U.S. provisional application Ser. No. 60/541,515, filed Feb. 2, 2004; and U.S. provisional application Ser. No. 60/532,598, filed Dec. 24, 2003. In addition, this application is a continuation-in-part of each of the following prior applications, the disclosures of each of which are hereby incorporated by reference in their entirety herein: International Application No. PCT/US2004/23881, filed Jul. 23, 2004; U.S. patent application Ser. No. 10/883,599, filed Jun. 30, 2004; U.S. patent application Ser. No. 10/773,618, filed Feb. 6, 2004; and U.S. patent application Ser. No. 10/773,792, filed Feb. 6, 2004.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made, in part, with government support under SBIR Grant No. 1 R43 CA 101421-01, awarded by the National Institutes of Health. The government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003] The field of this invention relates generally to novel polynucleotides and expression cassettes useful for expression of polypeptides, including heterologous polypeptides, in recombinant bacteria. In particular, this invention relates to recombinant bacteria comprising the novel expression cassettes and/or nucleic acid molecules which are useful in vaccine compositions.

BACKGROUND OF THE INVENTION

[0004] Microbes have begun to be developed for use as vaccines that deliver heterologous antigens. Heterologous antigen delivery is provided by microbes that have been modified to contain nucleic acid sequences encoding a protein or antigen originating from a different species. Heterologous antigen delivery is especially advantageous for treating or preventing diseases or conditions that result from especially virulent or lethal sources, such as cancer and pathogenic agents (for example, HIV or Hepatitis B), wherein injection of a native infectious agent or cancer cell is potentially deleterious to the recipient organism, and administration of attenuated or killed agent or cell has proven unsuccessful in eliciting an effective immune response, or where sufficient attenuation of the infectious

agent or cancer cell cannot be assured with acceptable certainty. Recently, certain bacterial strains have been developed as recombinant vaccines. For instance, an oral vaccine of attenuated *Salmonella* modified to express *Plasmodium berghei* circumsporozoite antigen has been shown to protect mice against malaria (Aggarwal et al. 1990. J. Exp. Med. 172:1083).

[0005] *Listeria monocytogenes* (*Listeria*) is a Gram-positive facultative intracellular bacterium that is being developed for use in antigen-specific vaccines due to its ability to prime a potent CD4+/CD8+ T-cell mediated response via both MHC class I and class II antigen presentation pathways. See, for instance, U.S. Pat. Nos. 6,051,237, 6,565,852, and 5,830,702.

[0006] *Listeria* has been studied for a number of years as a model for stimulating both innate and adaptive T cell-dependent antibacterial immunity. The ability of *Listeria* to effectively stimulate cellular immunity is based on its intracellular lifecycle. Upon infecting the host, the bacterium is rapidly taken up by phagocytes including macrophages and dendritic cells (DC) into a phagolysosomal compartment. The majority of the bacteria are subsequently degraded. Peptides resulting from proteolytic degradation of pathogens within phagosomes of infected APCs are loaded directly onto MHC class II molecules, and the processed antigens are expressed on the surface of the antigen presenting cell via the class II endosomal pathway, and these MHC II-peptide complexes activate CD4+ "helper" T cells that stimulate the production of antibodies. Within the acidic compartment, certain bacterial genes are activated including the cholesterol-dependent cytolysin, LLO, which can degrade the phagolysosome, releasing the bacterium into the cytosolic compartment of the host cell, where the surviving *Listeria* propagate. Efficient presentation of heterologous antigens via the MHC class I pathway requires de novo endogenous protein expression by *Listeria*. Within the cytoplasm of antigen presenting cells (APC), proteins synthesized and secreted by *Listeria* are sampled and degraded by the proteasome. The resulting peptides are shuttled into the endoplasmic reticulum by TAP proteins and loaded onto MHC class I molecules. The MHC I-peptide complex is delivered to the cell surface, which in combination with sufficient co-stimulation (signal 2) activates and stimulates cytotoxic T lymphocytes (CTLs) having the cognate T cell receptor to expand and subsequently recognize the MHC I-peptide complex displayed on, for example tumor cells. In the appropriate microenvironment, the activated T cell targets and kills the cancerous cell.

[0007] Given the mechanisms by which *Listeria* programs the presentation of heterologous antigens via the MHC class I pathway, the efficiency of both expression of heterologous genes and secretion of the newly synthesized protein from the bacterium into the cytoplasm of the infected (antigen presenting) cell is directly related to the potency of CD8+ T cell priming and/or activation. Since the level of Ag-specific T cell priming is directly related to vaccine efficacy, the efficiency of heterologous protein expression and secretion is linked directly to vaccine potency.

[0008] Thus, novel methods are needed in the art to optimize the efficiency of heterologous protein expression and secretion to maximize the potency of *Listeria*-based vaccines and other bacteria-based vaccines. It would also be

beneficial to optimize the efficiency of heterologous protein expression and secretion in bacterial host expression systems where expression and secretion of large quantities of heterologous protein is desired.

BRIEF SUMMARY OF THE INVENTION

[0009] The present invention generally provides novel polynucleotides including novel recombinant nucleic acid molecules, expression cassettes, and vectors for use in expressing and/or secreting polypeptides (e.g. heterologous polypeptides) in bacteria, especially *Listeria*. In some embodiments, these polynucleotides provide enhanced expression and/or secretion of polypeptides in bacteria. The present invention also generally provides bacteria comprising the recombinant nucleic acid molecules, expression cassettes, or vectors, as well as pharmaceutical, immunogenic, and vaccine compositions comprising the bacteria. These bacteria and compositions are useful in the induction of immune responses and in the treatment and/or prevention of a wide array of diseases or other conditions, including cancer, infections and autoimmunity.

[0010] In one aspect, the invention provides a recombinant nucleic acid molecule, comprising a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium, and a second polynucleotide encoding a polypeptide (e.g., an antigen), wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the second polynucleotide is also codon-optimized for expression in bacteria, such as *Listeria*. The invention also provides expression cassettes comprising this recombinant nucleic acid molecule and further comprising a promoter operably linked to the recombinant nucleic acid molecule. Vectors and bacteria comprising the recombinant nucleic acid molecules and/or expression cassette are also provided, as are pharmaceutical compositions, immunogenic compositions, and vaccines comprising the bacteria. Methods of using the bacteria or compositions comprising the bacteria to induce immune responses and/or to prevent or treat a condition such as a disease in a host are also provided.

[0011] In another aspect, the invention provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the bacterium. The invention also provides expression cassettes comprising this recombinant nucleic acid molecule and further comprising a promoter operably linked to the recombinant nucleic acid molecule. Vectors and bacteria comprising the recombinant nucleic acid molecule and/or expression cassette are also provided, as are pharmaceutical compositions, immunogenic compositions, and vaccines comprising the bacteria. Methods of using the bacteria or compositions

comprising the bacteria to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided.

[0012] In another aspect, the invention provides a recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in *Listeria*, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the polypeptide is foreign to the *Listeria* bacterium. In some embodiments, the signal peptide is native to *Listeria*. Pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the *Listeria* are also provided. Methods of using the *Listeria* (or compositions comprising the *Listeria*) to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided.

[0013] In another aspect, the invention provides a recombinant nucleic acid molecule, comprising a first polynucleotide encoding a non-secA1 bacterial signal peptide, and a second polynucleotide encoding a polypeptide (such as an antigen), wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide is heterologous to the signal peptide. In some embodiments, the first and/or second polynucleotides are codon-optimized for expression in bacteria, such as *Listeria*. The invention also provides expression cassettes comprising this recombinant nucleic acid molecule and further comprising a promoter operably linked to the recombinant nucleic acid molecule. Vectors and bacteria comprising the recombinant nucleic acid molecule and/or expression cassette are also provided, as are pharmaceutical compositions, immunogenic compositions, and vaccines comprising the bacteria. Methods of using the bacteria or compositions comprising the bacteria to induce immune responses and/or to treat a condition such as a disease in a host are also provided.

[0014] In still another aspect, the invention provides a recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide, and (b) a second polynucleotide encoding a polypeptide either heterologous to the signal peptide or foreign to the bacterium, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide or foreign to the bacterium (i.e., heterologous to the bacterium), or both. Pharmaceutical compositions, immunogenic compositions, and vaccines comprising the *Listeria* are also provided. Methods of using the *Listeria* (or compositions comprising

the *Listeria*) to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided.

[0015] In another aspect, the invention provides a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a polynucleotide encoding a polypeptide foreign to *Listeria* (e.g., a cancer or non-*Listerial* infectious disease antigen), wherein the polynucleotide encoding the foreign polypeptide is codon-optimized for expression in *Listeria*. In some embodiments, the recombinant nucleic acid molecule further comprises a polynucleotide that encodes a signal peptide in the same translational reading frame as the polynucleotide encoding the polypeptide foreign to *Listeria*. In some embodiments, the signal peptide is native to the *Listeria* bacterium. In other embodiments, the signal peptide is foreign to the *Listeria* bacterium. In some embodiments, the polynucleotide encoding the signal peptide is also codon-optimized for expression in *Listeria*. *Listeria* comprising the recombinant nucleic acid molecule are also provided. Pharmaceutical compositions, immunogenic compositions, and vaccines comprising the *Listeria* are also provided. In addition, the invention provides methods of using the recombinant *Listeria* bacteria to induce immune responses and/or to prevent or treat a condition (such as, but not limited to, a disease) in a host.

[0016] In another aspect, the invention provides a recombinant *Listeria* bacterium comprising an expression cassette, wherein the expression cassette comprises a polynucleotide encoding a polypeptide foreign to *Listeria* (e.g., a cancer or non-*Listerial* infectious disease antigen), wherein the polynucleotide encoding the foreign polypeptide is codon-optimized for expression in *Listeria*, and a promoter, operably linked to the polynucleotide encoding the foreign polypeptide. In some embodiments, the expression cassette further comprises a polynucleotide that encodes a signal peptide (a signal peptide either native or foreign to the *Listeria* bacterium) in the same translational reading frame as the polynucleotide encoding the polypeptide foreign to *Listeria* and operably linked to the promoter so that the expression cassette expresses a fusion protein comprising the signal peptide and the foreign polypeptide. In some embodiments, the polynucleotide encoding the signal peptide is also codon-optimized for expression in *Listeria*. Pharmaceutical compositions, immunogenic compositions, and vaccines comprising the *Listeria* are also provided. In addition, the invention provides methods of using the recombinant *Listeria* bacteria to induce immune responses and/or to prevent or treat a condition (e.g., a disease) in a host.

[0017] In another aspect, the invention provides a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-*Listerial* signal peptide, and (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-*Listerial* signal peptide and the polypeptide. The invention also provides an expression cassette comprising the recombinant nucleic acid molecule, wherein the expression cassette further comprises a promoter operably linked to the first and second polynucleotides of the recombinant nucleic acid molecule. Vectors comprising the recombinant nucleic acid molecule and/or the expression cassette are also provided. In addition,

a *Listeria* bacterium comprising the recombinant nucleic acid molecule and/or the expression cassette is also provided. Pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the *Listeria* bacterium are also provided. Methods of using the *Listeria* bacterium (or compositions comprising the *Listeria* bacterium) to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided.

[0018] In a further aspect, the invention provides a recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-*Listerial* signal peptide, and (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-*Listerial* signal peptide and the polypeptide. Pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the *Listeria* are also provided. Methods of using the *Listeria* (or compositions comprising the *Listeria*) to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided.

[0019] In still another aspect, the invention provides a *Listeria* bacterium (for instance, from the species *Listeria monocytogenes*) comprising an expression cassette which comprises a first polynucleotide encoding a non-*Listerial* signal peptide, a second polynucleotide encoding a polypeptide (e.g., an antigen) that is in the same translational reading frame as the first polynucleotide, and a promoter operably linked to both the first and second polynucleotides. The expression cassette encodes a fusion protein comprising both the non-*Listerial* signal peptide and the polypeptide. In some embodiments, the first and/or second polynucleotides are codon-optimized for expression in *Listeria*. Pharmaceutical compositions, immunogenic compositions, and vaccines comprising the *Listeria* are also provided. In addition, the invention provides methods of using the recombinant *Listeria* bacteria to induce immune responses and/or to prevent or treat a condition (e.g., a disease) in a host.

[0020] The invention also provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the polypeptide encoded by the second polynucleotide and the autolysin, or catalytically active fragment or catalytically active variant thereof, wherein, in the protein chimera, the polypeptide is fused to or is positioned within the autolysin, or catalytically active fragment or catalytically active variant thereof. Vectors and bacteria comprising the recombinant nucleic acid molecule and/or expression cassette are also provided, as are pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the bacteria. Methods of using the bacteria or compositions comprising the bacteria to induce immune responses and/or to treat a condition such as a disease in a host are also provided.

[0021] In another aspect, the invention provides a recombinant nucleic acid molecule, wherein the nucleic acid

molecule encodes at least two discrete non-Listerial polypeptides. The invention further provides an expression cassette comprising the recombinant nucleic acid molecule and further comprising a promoter, wherein the promoter is operably linked to the recombinant nucleic acid molecule. Vectors comprising the recombinant nucleic acid molecule and/or expression cassette are further provided. In addition a recombinant *Listeria* bacterium comprising the recombinant nucleic acid molecule (and/or the expression cassette) is also provided. Pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the *Listeria* are also provided. Methods of using the *Listeria* (or compositions comprising the *Listeria*) to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided.

[0022] In an additional aspect, the invention provides a recombinant *Listeria* bacterium comprising a polycistronic expression cassette, wherein the polycistronic expression cassette encodes at least two discrete non-Listerial polypeptides. Pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the *Listeria* are also provided. Methods of using the *Listeria* (or compositions comprising the *Listeria*) to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided.

[0023] In other aspects, the invention provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide, (b) a second polynucleotide encoding a secreted protein, or a fragment thereof, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a third polynucleotide encoding a polypeptide heterologous to the secreted protein, or fragment thereof, wherein the third polynucleotide is in the same translational reading frame as the first and second polynucleotides, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the third polynucleotide, and the secreted protein, or fragment thereof, and wherein the polypeptide encoded by the third polynucleotide is fused to the secreted protein, or fragment thereof, or is positioned within the secreted protein, or fragment thereof, in the protein chimera. An expression cassette comprising the recombinant nucleic acid molecule and further comprising a promoter operably linked to the first, second, and third polynucleotides of the recombinant nucleic acid molecule is also provided. Vectors and bacteria comprising the recombinant nucleic acid molecule and/or expression cassette are also provided, as are pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the bacteria. Methods of using the bacteria or compositions comprising the bacteria to induce an immune response and/or to prevent or treat a condition in a host are also provided.

[0024] In some embodiments, the methods of inducing an immune response in a host to an antigen comprise administering to the host an effective amount of a composition comprising a recombinant bacterium described herein (e.g., in any of the aspects above, or in the Detailed Description of the Invention or Examples, below) to the host, wherein a polypeptide encoded by the recombinant nucleic acid molecule, expression cassette, and/or vector in the bacterium comprises the antigen. In some embodiments, the methods of preventing or treating a condition, such as a disease, in a

host comprise administering to the host an effective amount of a composition comprising a recombinant bacterium described herein to the host.

[0025] The invention further provides the use of a recombinant bacterium described herein (e.g., in any of the aspects above, or in the Detailed Description of the Invention or Examples, below) in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein a polypeptide encoded by the recombinant nucleic acid molecule, expression cassette, and/or vector in the bacterium comprises the antigen. In some embodiments, the antigen is a heterologous antigen. The invention also provides the use of a recombinant bacterium described herein in the manufacture of a medicament for preventing or treating a condition in a host (e.g., a disease such as cancer or an infectious disease). The invention further provides the recombinant bacteria described herein for use in inducing an immune response in a host to an antigen, wherein a polypeptide encoded by the recombinant nucleic acid molecule, expression cassette, and/or vector in the bacterium comprises the antigen. The invention further provides the recombinant bacteria described herein for use in the prevention or treatment of a condition (such as a disease) in a host.

[0026] In further aspects, the invention provides improved methods of expressing and secreting heterologous proteins in host bacteria.

[0027] Methods of making bacteria comprising each of the recombinant nucleic acid molecules and expression cassettes described above are also provided. Methods of using the bacteria to produce vaccines are also provided.

[0028] The invention further provides a variety of polynucleotides encoding signal peptides and/or antigens, including the polynucleotides which have been codon-optimized for expression in *Listeria monocytogenes*.

DRAWINGS

[0029] FIG. 1 shows the hly promoter alignment for the *Listeria monocytogenes* DP-1 A056 (SEQ ID NO:1) (bottom sequence) and EGD strains (SEQ ID NO:2) (top sequence).

[0030] FIG. 2 shows the sequence (SEQ ID NO:3) of a polynucleotide encoding a fusion protein comprising the LLO signal peptide, LLO PEST sequence, and the full-length human EphA2 antigen.

[0031] FIG. 3 shows the sequence (SEQ ID NO:4) of the fusion protein encoded by the polynucleotide shown in FIG. 2.

[0032] FIG. 4 shows the native nucleotide sequence (SEQ ID NO:5) that encodes the human EphA2 extracellular domain (EX2).

[0033] FIG. 5 shows a nucleotide sequence (SEQ ID NO:6) encoding the human EphA2 extracellular domain that has been codon-optimized for expression in *Listeria monocytogenes*.

[0034] FIG. 6 shows the amino acid sequence (SEQ ID NO:7) of the human EphA2 extracellular domain (EX2).

[0035] FIG. 7 shows a non-codon optimized polynucleotide sequence (SEQ ID NO:8) encoding a fusion protein comprising an LLO signal peptide, LLO PEST sequence and the extracellular domain of human EphA2.

[0036] FIG. 8 shows the sequence (SEQ ID NO:9) of the fusion protein encoded by the coding sequence shown in FIG. 7.

[0037] FIG. 9 shows an expression cassette (SEQ ID NO:10) comprising the hly promoter and encoding a fusion protein comprising an LLO signal peptide, LLO PEST sequence and the extracellular domain of human EphA2. In this sequence, the sequence encoding the human EphA2 extracellular domain is codon-optimized for expression in *Listeria monocytogenes*.

[0038] FIG. 10 shows the amino acid sequence (SEQ ID NO:11) encoded by the expression cassette of FIG. 9.

[0039] FIG. 11 shows an expression cassette (SEQ ID NO:12) comprising the hly promoter and encoding a fusion protein comprising an LLO signal peptide, LLO PEST sequence and the extracellular domain of human EphA2. In this sequence, the sequences encoding the LLO signal peptide, LLO PEST, and human EphA2 extracellular domain have all been codon-optimized for expression in *Listeria monocytogenes*.

[0040] FIG. 12 shows the amino acid sequence (SEQ ID NO:13) encoded by the expression cassette of FIG. 11.

[0041] FIG. 13 shows an expression cassette (SEQ ID NO:14) comprising the hly promoter and encoding a fusion protein comprising the phoD Tat signal peptide and the extracellular domain of human EphA2. In this sequence, the sequences encoding the phoD Tat signal peptide and human EphA2 extracellular domain have both been codon-optimized for expression in *Listeria monocytogenes*.

[0042] FIG. 14 shows the amino acid sequence (SEQ ID NO:15) encoded by the expression cassette of FIG. 13.

[0043] FIG. 15 shows the native nucleotide sequence (SEQ ID NO:16) that encodes the human EphA2 intracellular domain (CO).

[0044] FIG. 16 shows a nucleotide sequence (SEQ ID NO:17) encoding the human EphA2 intracellular domain that has been codon-optimized for expression in *Listeria monocytogenes*.

[0045] FIG. 17 shows the amino acid sequence (SEQ ID NO:18) of the human EphA2 intracellular domain (EX2).

[0046] FIG. 18 shows a non-codon optimized polynucleotide sequence (SEQ ID NO:19) encoding a fusion protein comprising an LLO signal peptide, LLO PEST sequence and the intracellular domain of human EphA2.

[0047] FIG. 19 shows the sequence (SEQ ID NO:20) of the fusion protein encoded by the coding sequence shown in FIG. 18.

[0048] FIG. 20 shows an expression cassette (SEQ ID NO:21) comprising the hly promoter and encoding a fusion protein comprising an LLO signal peptide, LLO PEST sequence and the intracellular domain of human EphA2. In this sequence, the sequence encoding the human EphA2 intracellular domain is codon-optimized for expression in *Listeria monocytogenes*.

[0049] FIG. 21 shows the amino acid sequence (SEQ ID NO:22) encoded by the expression cassette of FIG. 20.

[0050] FIG. 22 shows an expression cassette (SEQ ID NO:23) comprising the hly promoter and encoding a fusion protein comprising an LLO signal peptide, LLO PEST sequence and the intracellular domain of human EphA2. In this sequence, the sequences encoding the LLO signal peptide, LLO PEST, and human EphA2 intracellular domain have all been codon-optimized for expression in *Listeria monocytogenes*.

[0051] FIG. 23 shows the amino acid sequence encoded (SEQ ID NO:24) by the expression cassette of FIG. 22.

[0052] FIG. 24 shows an expression cassette (SEQ ID NO:25) comprising the hly promoter and encoding a fusion protein comprising a phoD Tat signal peptide and the intracellular domain of human EphA2. In this sequence, the sequences encoding both the phoD Tat signal peptide and human EphA2 intracellular domain have been codon-optimized for expression in *Listeria monocytogenes*.

[0053] FIG. 25 shows the amino acid sequence (SEQ ID NO:26) encoded by the expression cassette of FIG. 24.

[0054] FIG. 26 shows a codon-optimized expression cassette (SEQ ID NO:27) comprising the hly promoter and encoding a fusion protein comprising an LLO signal peptide and the NY-ESO-1 antigen. Both the sequences encoding the signal peptide and the antigen are codon-optimized for expression in *Listeria monocytogenes*.

[0055] FIG. 27 shows the amino acid sequence (SEQ ID NO:28) encoded by the expression cassette of FIG. 26.

[0056] FIG. 28 shows a polynucleotide (SEQ ID NO:29) comprising the hly promoter operably linked to a codon-optimized sequence encoding a Usp45 signal peptide.

[0057] FIG. 29 shows a polynucleotide (SEQ ID NO:30) comprising the hly promoter operably linked to a native sequence encoding a p60 signal peptide.

[0058] FIG. 30 shows a polynucleotide (SEQ ID NO:31) comprising the hly promoter operably linked to a codon-optimized sequence encoding a p60 signal peptide.

[0059] FIG. 31 shows the sequence (SEQ ID NO:32) of an hlyP-p60 gene fragment.

[0060] FIG. 32 (includes FIG. 32A, 32B, and 32C) shows the sequence (SEQ ID NO:33) of pAM401-MCS, the pAM401 plasmid containing a multiple cloning site (MCS) from pPL2 vector.

[0061] FIG. 33 shows the coding sequence (SEQ ID NO:34) for human mesothelin which has been codon-optimized for expression in *Listeria monocytogenes*.

[0062] FIG. 34 shows the amino acid sequence of human mesothelin (SEQ ID NO:35).

[0063] FIG. 35 shows the coding sequence (SEQ ID NO:36) for murine mesothelin which has been codon-optimized for expression in *Listeria monocytogenes*.

[0064] FIG. 36 shows the amino acid sequence (SEQ ID NO:37) of murine mesothelin.

[0065] FIG. 37 shows a Western blot analysis of secreted protein from recombinant *Listeria* encoding a native EphA2 CO domain sequence.

[0066] FIG. 38 shows a Western blot analysis of secreted protein from recombinant *Listeria* encoding native or codon-optimized LLO secA1 signal peptide fused with codon-optimized EphA2 EX2 domain sequence.

[0067] FIG. 39 shows a Western blot analysis of secreted protein from recombinant *Listeria* encoding native or codon-optimized LLO secA1 signal peptide or codon-optimized Tat signal peptide fused with codon-optimized EphA2 CO domain sequence.

[0068] FIG. 40 shows a Western blot analysis of lysate from 293 cells 48 hr following transfection with pCDNA4 plasmid DNA encoding full-length native EphA2 sequence.

[0069] FIG. 41 is a graph showing that immunization of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors with recombinant *Listeria* encoding OVA.AH1 or OVA.AH1-A5 confers long-term survival.

[0070] FIG. 42 is a graph showing the increased survival of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors when immunized with recombinant *Listeria* encoding codon-optimized secA1 signal peptide fused with codon-optimized EphA2 EX2 domain sequence.

[0071] FIG. 43 is a graph showing that immunization of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors with recombinant *Listeria* encoding EphA2 CO domain confers long-term survival.

[0072] FIG. 44 is a graph showing that immunization of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors with recombinant *Listeria* encoding EphA2 CO domain but not with plasmid DNA encoding full-length EphA2 confers long-term survival.

[0073] FIG. 45 is a graph showing that *Listeria* expressing hEphA2 elicits an EphA2 specific CD8+ T cell response.

[0074] FIG. 46 is a graph showing that both CD4+ and CD8+ T cell responses contribute to the hEphA2-directed anti-tumor efficacy of *Listeria* expressing hEphA2.

[0075] FIG. 47 shows the sequence (SEQ ID NO:38) of the *Listeria monocytogenes* strain 10403S hly promoter operably linked to Protective Antigen signal peptide from *B. anthracis*, codon-optimized for secretion in *Listeria monocytogenes*. Six additional nucleotides (5'-GGAATCC-3') corresponding to the Bam HI restriction enzyme recognition site were included at the carboxy terminus of the signal peptide sequence, facilitating operable in-frame linkage to any selected coding sequence. The 5' end of the hly promoter contains a unique Kpn I restriction enzyme recognition site.

[0076] FIG. 48 shows the efficient expression and secretion of full-length human tumor antigens from recombinant *Listeria*. FIG. 48A shows mesothelin expression/secretion with constructs consisting of LLO signal peptide fused with human mesothelin, using native codons. FIG. 48B shows mesothelin expression/secretion with constructs comprising various signal peptides fused with human mesothelin codon-optimized for expression in *Listeria*. FIG. 48C shows the expression/secretion of NY-ESO-1 with constructs comprising codon-optimized LLO signal peptide fused with human mesothelin codon-optimized NY-ESO-1.

[0077] FIG. 49 shows the coding sequences of pEphA2KD (SEQ ID NO:39).

[0078] FIG. 50 shows the Mlu I subfragment (SEQ ID NO:40) of codon-optimized human EphA2 containing the actA-plcB intergenic region.

[0079] FIG. 51 shows the sequence (SEQ ID NO:41) of the hly promoter-70 N-terminal p60 amino acids.

[0080] FIG. 52 shows the KpnI-BamHI sub-fragment (SEQ ID NO:42) of plasmid pPL2-hlyP-Np60 CodOp(1-77).

[0081] FIG. 53 shows the KpnI-BamHI sub-fragment (SEQ ID NO:43) of plasmid pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin.

[0082] FIG. 54 shows the KpnI-BamHI sub-fragment (SEQ ID NO:44) of plasmid pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin ΔSP/ΔGPI.

[0083] FIG. 55 shows the Western blot analysis of the expression and secretion of antigens from recombinant *Listeria* comprising antigen-bacterial protein chimeras.

[0084] FIG. 56 shows the Western blot analysis of the expression of the intracellular domain (ICD) of EphA2 from a bicistronic message.

[0085] FIG. 57 shows the Western blot analysis of the plasmid based expression and secretion of murine mesothelin as a function of N-terminal fusion with various codon-optimized signal peptides as evidenced in different bacterial fractions: secreted protein (FIG. 57A); cell wall (FIG. 57B); and cell lysate (FIG. 57C).

[0086] FIG. 58 shows the Western blot analysis of chromosomal-based expression and secretion of human mesothelin in *Listeria monocytogenes*. Western blot analysis of mesothelin expression in various bacterial cell fractions, with results from control *Listeria* (not encoding mesothelin) and *Listeria* encoding mesothelin expressed from the indicated signal sequences, is shown.

[0087] FIGS. 59A and 59B are graphs showing the delivery of a heterologous antigen (AH1-A5) to MHC Class I pathway by a *Listeria* vaccine. The *Listeria* vaccine comprised *Listeria* expressing a p60-AH1-A5 protein chimera (AH1-A5 embedded in p60) (FIG. 59A) or *Listeria* expressing a fusion protein comprising an LLO signal peptide and AH1-A5 (FIG. 59B).

[0088] FIGS. 60A and 60B are graphs showing the *Listeria* vaccine mediated delivery of bacteria-specific antigens to MHC Class I pathway, where the vaccine comprised *Listeria* expressing a p60-AH1-A5 protein chimera (AH1-A5 embedded in p60) (FIG. 60A) or *Listeria* expressing a fusion protein comprising an LLO signal peptide and AH1-A5 (FIG. 60B), and where the test peptides added to the cell based assay were no test peptide (unstimulated) (FIG. 60A), LLO₉₁₋₉₉ (FIG. 60A), no test peptide (FIG. 60B), or p60₂₁₇₋₂₂₅ (FIG. 60B).

[0089] FIG. 61 is a graph showing the therapeutic efficacy of *Listeria* expressing human mesothelin in vaccinated tumor-bearing animals, where tumor cells were engineered to express human mesothelin.

[0090] FIG. 62 is a graph showing the reduction in lung tumor nodule level in tumor-bearing mice vaccinated with *Listeria* expressing human mesothelin, where the tumor cells were engineered to express human mesothelin.

[0091] FIG. 63 is a graph showing a control study using CT.26 parental target cells, i.e., cells not engineered to express human mesothelin, that demonstrates the anti-tumor efficacy of Lm-Meso vaccination is mesothelin specific.

[0092] FIG. 64 is a graph showing that vaccination with *Listeria* expressing codon optimized human mesothelin reduces tumor volume.

[0093] FIG. 65 shows the results of ELISPOT experiments which show the immunogenicity of a *Listeria* Δ actA/ Δ inlB-hMesothelin strain where the nucleic acid encoding hMesothelin has been integrated into the *Listeria* genome.

DETAILED DESCRIPTION OF THE INVENTION

[0094] I. Introduction

[0095] The present invention provides a variety of polynucleotides including recombinant nucleic acid molecules, expression cassettes, and expression vectors useful for expression and/or secretion of polypeptides, including heterologous polypeptides (e.g. antigens and/or mammalian proteins), in bacteria, such as *Listeria*. In some embodiments, these polynucleotides can be used for enhanced expression and/or secretion of polypeptides in bacteria. Some of the expression cassettes comprise codon-optimized coding sequences for the polypeptide and/or for the signal peptide. In addition, some of the expression cassettes for use in bacteria contain signal peptide sequences derived from other bacterial sources and/or from a variety of different secretory pathways. Bacteria comprising the expression cassettes are also provided, as are compositions, such as vaccines, containing the bacteria. Methods of using the polynucleotides, bacteria, and compositions to induce an immune response and/or to prevent or treat a condition, such as a disease (e.g. cancer), in a host are also provided.

[0096] The invention is based, in part, on the discovery that codon-optimization of the signal peptide sequence in an expression cassette enhances the expression and/or secretion of a heterologous polypeptide (such as an antigen) from recombinant bacteria (particularly in combination with codon-optimization of the heterologous polypeptide), even when the signal peptide sequence is native to the bacteria (see, e.g., Examples 19 and 27, below). Additionally, it has been discovered that signal peptide sequences from non-secA1 secretory pathways and/or signal peptide sequences from non-*Listeria* bacterial sources can also be used to effect efficient expression and/or secretion of heterologous polypeptides from *Listeria* (see, e.g., Examples 19, 27, and 30 below). The invention is also based, in part, on the additional discovery that codon-optimization of the coding sequences of heterologous polypeptides enhances expression and/or secretion of the heterologous polypeptides in *Listeria* (see e.g., Example 19, below). Enhanced expression and/or secretion of the heterologous protein obtained through optimization of the expression cassette has also been shown to lead to enhanced immunogenicity of the bacteria comprising the optimized expression cassettes (see, e.g., Example 20, below). In addition, expression cassettes encoding protein chimeras comprising a heterologous antigen embedded within an autolysin have been shown to be useful in effecting efficient expression and secretion of a heterologous antigen in *Listeria* (see, e.g., Example 29, below). The autolysin protein chimeras have also been

shown to be immunogenic (see, e.g., Example 31A, below). In addition, *Listeria* comprising codon-optimized expression cassettes and/or expression cassettes comprising non-*Listeria* signal peptides have also been shown to be immunogenic, reduce tumor volume, and increase survival in a mouse model (see, e.g., Examples 31B-E, below).

[0097] Accordingly, in one aspect, the invention provides a recombinant nucleic acid molecule, comprising a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium, and a second polynucleotide encoding a polypeptide (e.g., an antigen), wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the second polynucleotide is codon-optimized as well (typically for expression in the same type of bacteria as the first polynucleotide). In some embodiments, the first polynucleotide or the first and second polynucleotides are codon-optimized for expression in *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria or *E. coli*. In some embodiments, the polynucleotide(s) is codon-optimized for expression in *Listeria*, such as *Listeria monocytogenes*. In some embodiments, the polypeptide encoded by the second polynucleotide is (or comprises) an antigen, which, in some instances, may be a non-bacterial antigen. For instance, the antigen is, in some embodiments a tumor-associated antigen or is derived from such a tumor-associated antigen. For instance, in some embodiments, the antigen is K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA, or is derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. For instance, in some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant of mesothelin. In some other embodiments, the antigen is NY-ESO-1, or an antigenic fragment or antigenic variant of NY-ESO-1. In some embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, the signal peptide is bacterial (*Listeria* or non-*Listeria*). In some embodiments, the signal peptide encoded by the codon-optimized first polynucleotide is native to the bacterium. In other embodiments, the signal peptide encoded by the codon-optimized first polynucleotide is foreign to the bacterium. In some embodiments, the signal peptide is a secA1 signal peptide, such as an LLO signal peptide from *Listeria monocytogenes*, a Usp45 signal peptide from *Lactococcus lactis*, or a Protective Antigen signal peptide from *Bacillus anthracis*. In some embodiments, the signal peptide is a secA2 signal peptide. For instance, the signal peptide may be the p60 signal peptide from *Listeria monocytogenes*. In addition, the recombinant nucleic acid molecule optionally comprises a third polynucleotide sequence encoding p60, or a fragment thereof, in the same translational reading frame as the first and second polynucleotides, wherein the second polynucleotide is positioned within the third polynucleotide or between the first and third polynucleotides. In still further embodiments, the signal peptide is a Tat signal peptide, such as a *B. subtilis* Tat signal peptide (e.g., PhoD). The invention also provides expression cassettes comprising the recombinant nucleic acid molecule and further comprising a pro-

moter operably linked to the recombinant nucleic acid molecule (e.g., to the first and second polynucleotides (and third polynucleotide, if present)). Expression vectors and recombinant bacteria (e.g. *Listeria*) comprising the expression cassette are also provided, as are pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the bacteria. Methods of using the bacteria or compositions comprising the bacteria to induce an immune response and/or prevent or treat a condition, such as a disease, are also provided. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0098] In a second aspect, the invention provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the second polynucleotide is heterologous to the first polynucleotide. In some embodiments, the polypeptide is foreign to the bacterium to which the signal peptide is native. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide, foreign to the bacterium, or both. In some embodiments, the bacterium from which the signal peptide is derived is an intracellular bacterium. In some embodiments, the bacterium is selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria and *E. coli*. In some embodiments the bacterium is a *Listeria* bacterium (e.g., *Listeria monocytogenes*). In some embodiments, second polynucleotide is codon-optimized for expression in the bacterium. In some embodiments, the codon-optimization of the first and/or second polynucleotide enhances expression in and/or secretion from the bacterium of the encoded fusion protein (relative to the non-codon-optimized sequence). In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen. The polypeptide encoded by the second polynucleotide is an antigen. In some embodiments, the antigen is a non-bacterial antigen. In some embodiments, the antigen is a tumor-associated antigen or comprises an antigen derived from a tumor-associated antigen. In some embodiments, the antigen is selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or is derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA. For instance, in some embodiments, the antigen is mesothelin, or an antigenic fragment or variant thereof, or is NY-ESO-1, or an antigenic fragment or variant thereof. In some alternative embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, the signal peptide is a secA1 signal peptide (e.g., LLO signal peptide from *Listeria monocytogenes*). In

some embodiments, the signal peptide is a secA2 signal peptide (e.g., p60 signal peptide from *Listeria monocytogenes*). An expression cassette comprising the recombinant nucleic acid molecule and further comprising a promoter operably linked to the first and second polynucleotides of the recombinant nucleic acid molecule is also provided. An expression vector comprising the expression cassette is also provided. A recombinant bacterium comprising the recombinant nucleic acid molecule, wherein the first polynucleotide is codon-optimized for expression in the recombinant bacterium is also provided. In some embodiments, the recombinant bacterium is an intracellular bacterium. In some embodiments, the recombinant bacterium is selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria and *E. coli*. In some embodiments, the bacterium is a recombinant *Listeria* bacterium (e.g., a recombinant *Listeria monocytogenes* bacterium). An immunogenic composition comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is an antigen is further provided. Methods of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is (or comprises) the antigen, are also provided. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0099] In a third aspect, the invention provides a recombinant *Listeria* bacterium (e.g., *Listeria monocytogenes*) comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in the *Listeria* bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the recombinant nucleic acid molecule is part of an expression cassette that further comprises a promoter operably linked to both the first and second polynucleotides. In other words, in some embodiments the recombinant *Listeria* bacterium comprises an expression cassette which comprises the recombinant nucleic acid molecule, wherein the expression cassette further comprises a promoter operably linked to both the first and second polynucleotides of the recombinant nucleic acid molecule. In some embodiments, the expression cassette is a polycistronic expression cassette. In some embodiments, the second polynucleotide is codon-optimized for expression in the *Listeria* bacterium. In some embodiments, the codon-optimization of the first and/or second polynucleotide enhances expression in and/or secretion from the *Listeria* bacterium of the encoded fusion protein (relative to the non-codon-optimized sequence). In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the *Listeria* bacterium (i.e., heterologous to the *Listeria* bacterium). In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen (e.g., a non-*Listeria* or non-bacterial antigen). In some embodi-

ments, the polypeptide encoded by the second polynucleotide is an antigen. In some embodiments, the antigen is a tumor-associated antigen or is derived from a tumor-associated antigen. In some embodiments, the antigen is selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or is derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA. For instance, in some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant thereof. In some embodiments, the antigen is human mesothelin. In some embodiments, the antigen is human mesothelin deleted of its signal peptide and GPI linker domain. In some alternative embodiments, the antigen is NY-ESO-1, or an antigenic fragment or antigenic variant thereof. In some alternative embodiments, the antigen is an infectious disease antigen or is an antigen derived from an infectious disease antigen. In some embodiments, the signal peptide is non-*Listeria*. In some embodiments, the signal peptide is bacterial. In some embodiments, the signal peptide is foreign to the *Listeria* bacterium. In other embodiments, the signal peptide is native to the *Listeria* bacterium. In some embodiments, the signal peptide is a secA1 signal peptide (e.g., LLO signal peptide from *Listeria monocytogenes*, Usp45 signal peptide from *Lactococcus lactis*, and Protective Antigen signal peptide from *Bacillus anthracis*). In some embodiments, the signal peptide is a secA2 signal peptide (e.g., p60 signal peptide from *Listeria monocytogenes*). In some embodiments, the signal peptide is a Tat signal peptide (e.g., PhoD signal peptide from *B. subtilis*). In some embodiments, the *Listeria* bacterium is attenuated. For instance, the *Listeria* may be attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the recombinant *Listeria* bacterium is deficient with respect to ActA, Internalin B, or both Act A and Internalin B (e.g., an Δ ActA Δ inlB double deletion mutant). In some embodiments, the recombinant *Listeria* bacterium is deleted in functional ActA, Internalin B, or both Act A and Internalin B. In some embodiments, the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound (e.g., a psoralen compound). The invention also provides a pharmaceutical composition comprising the recombinant *Listeria* bacterium and a pharmaceutically acceptable carrier, as well as an immunogenic composition comprising the recombinant *Listeria* bacterium, wherein the polypeptide encoded by the second polynucleotide is an antigen. The invention also provides a vaccine comprising the recombinant *Listeria* bacterium. Methods of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is (or comprises) an antigen are also provided. Also provided are methods of preventing or treating a condition (e.g., a disease such as cancer or an infectious disease) in a host comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0100] In a fourth aspect, the invention provides a recombinant nucleic acid molecule, comprising a first polynucleotide encoding a non-secA1 bacterial signal peptide, and a second polynucleotide encoding a polypeptide (e.g., an antigen), wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the first polynucleotide and/or the second polynucleotide is codon-optimized for expression in a particular type of bacterium. In some embodiments, the codon-optimization of the first and/or second polynucleotide enhances expression in and/or secretion from the bacterium of the fusion protein (relative to the non-codon-optimized sequence). In some embodiments, the first polynucleotide and/or the second polynucleotide is codon-optimized for expression in *Listeria*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, *Mycobacteria* or *E. coli*. In some embodiments, the polynucleotide(s) is codon-optimized for expression in *Listeria*, such as *Listeria monocytogenes*. In some embodiments, the signal peptide encoded by the codon-optimized first polynucleotide is native to the bacterium for which it is codon-optimized. In some embodiments, the first polynucleotide encoding the signal peptide is heterologous to the second polynucleotide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen. In some embodiments, the polypeptide encoded by the second polynucleotide is an antigen, which, in some instances, may be a non-bacterial antigen. In some embodiments, the antigen is a tumor-associated antigen or is derived from such a tumor-associated antigen. For instance, in some embodiments, the antigen is K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA, or is derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. For instance, in some embodiments, the antigen is mesothelin, or is an antigenic fragment or antigenic variant of mesothelin. In some other embodiments, the antigen is NY-ESO-1, or an antigenic fragment or antigenic variant of NY-ESO-1. In some embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, the signal peptide encoded by the first polynucleotide of the recombinant nucleic acid molecule is *Listeria*. In other embodiments, the signal peptide is non-*Listeria*. In some embodiments, the signal peptide is derived from a gram positive bacterium. In some embodiments, the signal peptide is derived from a bacterium belonging to the genus *Bacillus*, *Staphylococcus*, or *Lactococcus*. In some embodiments, the signal peptide is a secA2 signal peptide. For instance, the signal peptide may be the p60 signal peptide from *Listeria monocytogenes*. In addition, the recombinant nucleic acid molecule optionally comprises a third polynucleotide sequence encoding p60, or a fragment thereof, in the same translational reading frame as the first and second polynucleotides, wherein the second polynucleotide is positioned within the third polynucleotide or between the first and third polynucleotides. In still further embodiments, the signal peptide is a Tat signal peptide, such as a *B. subtilis* Tat signal peptide (e.g., a *B. subtilis* PhoD

signal peptide). The invention also provides expression cassettes comprising the recombinant nucleic acid molecule and further comprising a promoter operably linked to the first and second polynucleotides of the recombinant nucleic acid molecule. Expression vectors and bacteria comprising the expression cassette and/or recombinant nucleic acid molecule are also provided, as are pharmaceutical compositions, immunogenic compositions, and vaccines, comprising the bacteria. In some embodiments, the recombinant bacterium comprising the expression cassette or recombinant nucleic acid molecule is an intracellular bacterium. In some embodiments, the bacterium is a bacterium selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria or *E. coli*. In some embodiments, the bacterium is a *Listeria* bacterium (e.g., a member of the species *Listeria monocytogenes*). In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the bacterium (i.e., heterologous to the bacterium). Methods of using the bacteria or compositions comprising the bacteria to induce an immune response and/or to prevent or treat a condition (e.g., a disease) in a host are also provided. In some embodiment, the condition is cancer. In other embodiments, the condition is an infectious disease. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0101] In another aspect, the invention provides a recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide or is foreign to the bacterium, or both. In some embodiments, the *Listeria* bacterium belongs to the species *Listeria monocytogenes*. In some embodiments, the recombinant nucleic acid molecule is part of an expression cassette that further comprises a promoter operably linked to both the first and second polynucleotides. In other words, in some embodiments, the recombinant *Listeria* bacterium comprises an expression cassette which comprises the recombinant nucleic acid molecule, wherein the expression cassette further comprises a promoter operably linked to both the first and second polynucleotides of the recombinant nucleic acid molecule. In some embodiments, the expression cassette is a polycistronic expression cassette. In some embodiments, the first polynucleotide, the second polynucleotide, or both the first and second polynucleotide are codon-optimized for expression in the *Listeria* bacterium (e.g., *Listeria monocytogenes*). In some embodiments, the codon-optimization of the first and/or second polynucleotide enhances expression in and/or secretion from the bacterium of the fusion protein (relative to the non-codon-optimized sequence). In some embodiments, the first and second polynucleotides are heterologous to each other. In some embodiments, the polypeptide encoded by the second polynucleotide and the signal peptide are heterologous to each other. In some embodi-

ments, the polypeptide encoded by the second polynucleotide is foreign to the *Listeria* bacterium (i.e., heterologous to the *Listeria* bacterium). In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen. In some embodiments, the polypeptide encoded by the second polynucleotide is an antigen (e.g., a non-*Listeria* or non-bacterial antigen). In some embodiments, the antigen is a tumor-associated antigen or is derived from a tumor-associated antigen. In some embodiments, the antigen is selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or is derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA. For instance, in some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant thereof. In some embodiments, the antigen is human mesothelin. In some embodiments, the antigen is human mesothelin deleted of its signal peptide and GPI linker domain. In some alternative embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, the signal peptide is non-*Listeria*. In some embodiments, the non-secA1 signal peptide is a *Listeria* signal peptide. In other embodiments, the non-secA1 signal peptide is a non-*Listeria* signal peptide. In some embodiments, the signal peptide is a secA2 signal peptide (e.g., p60 signal peptide from *Listeria monocytogenes*). In some embodiments, the recombinant nucleic acid molecule comprising a secA2 signal peptide, further comprises a third polynucleotide encoding a secA2 autolysin (e.g., p60 or N-acetylmuramidase), or a fragment thereof (e.g., a catalytically active fragment), in the same translational reading frame as the first and second polynucleotides, wherein the second polynucleotide is positioned within the third polynucleotide or between the first and third polynucleotides of the recombinant nucleic acid molecule. In some embodiments, the second polynucleotide is positioned within the third polynucleotide. In some embodiments, the signal peptide is a Tat signal peptide. In some embodiments, the signal peptide is a Tat signal peptide derived *B. subtilis* (e.g., PhoD signal peptide from *B. subtilis*). In some embodiments, the *Listeria* bacterium is attenuated. For instance, the *Listeria* may be attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the recombinant *Listeria* bacterium is deficient with respect to ActA, Internalin B, or both Act A and Internalin B (e.g., an Δ actA Δ inlB double deletion mutant). In some embodiments, the recombinant *Listeria* bacterium is deleted in functional ActA, Internalin B, or both Act A and Internalin B. In some embodiments, the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound (e.g., a psoralen compound). The invention also provides a pharmaceutical composition comprising the recombinant *Listeria* bacterium and a pharmaceutically acceptable carrier. The invention also provides an immunogenic composition comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is an antigen. The invention also provides a vaccine comprising the recombinant *Listeria* bacterium. Methods of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bac-

terium, wherein the polypeptide encoded by the second polynucleotide is (or comprises) an antigen are also provided. Also provided are methods of preventing or treating a condition (e.g., a disease such as cancer or an infectious disease) in a host comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0102] In another aspect, the invention provides a recombinant nucleic acid molecule comprising a polynucleotide encoding a polypeptide foreign to a *Listeria* bacterium (such as an antigen like a cancer antigen or a non-*Listeria* bacterial antigen), wherein the polynucleotide is codon-optimized for expression in *Listeria*. In some embodiments, the codon-optimization of the polynucleotide enhances expression in and/or secretion from a *Listeria* bacterium of the polypeptide (relative to the non-codon-optimized sequence). In some embodiments, the foreign polypeptide comprises an antigen. In some embodiments, the foreign polypeptide is an antigen. In some embodiments, the antigen is a non-bacterial antigen. For instance, the antigen is, in some embodiments a tumor-associated antigen or is derived from such a tumor-associated antigen. For instance, in some embodiments, the polypeptide is K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA, or is derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. In some embodiments, the antigen is mesothelin, or is an antigenic fragment or antigenic variant of mesothelin. In some other embodiments, the antigen is NY-ESO-1, or is an antigenic fragment or variant of NY-ESO-1. In some other embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, the recombinant nucleic acid molecule further comprises a polynucleotide encoding a signal peptide in the same translational frame as the foreign polypeptide so that the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the foreign polypeptide. In some embodiments, the polynucleotide encoding the signal peptide (which may or may not be native to *Listeria*) is codon-optimized for expression in *Listeria monocytogenes*. The invention further provides an expression cassette comprising the recombinant nucleic acid molecule and further comprising a promoter operably linked to the first and second polynucleotides of the recombinant nucleic acid molecule. A vector (e.g., an expression vector) comprising the recombinant nucleic acid molecule and/or expression cassette is also provided. The invention also provides a recombinant *Listeria* bacterium comprising the recombinant nucleic acid molecule and/or expression cassette. In some embodiments, the *Listeria* bacterium belongs to the species *Listeria monocytogenes*. Pharmaceutical compositions, immunogenic compositions, and vaccines comprising the recombinant *Listeria* bacteria are also provided. The invention further provides a method of inducing an immune response in host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium, wherein the polypeptide is (or comprises) the antigen. In addition, the

invention provides methods of using the recombinant *Listeria* bacteria to induce an immune response and/or prevent or treat a condition (e.g., a disease). The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the foreign polypeptide comprises the antigen is also provided.

[0103] In another aspect, the invention provides a recombinant *Listeria* bacterium comprising an expression cassette, wherein the expression cassette comprises a polynucleotide encoding a polypeptide foreign to the *Listeria* bacterium (such as an antigen like a cancer antigen or a non-*Listeria* bacterial antigen), wherein the polynucleotide is codon-optimized for expression in *Listeria*, and a promoter, operably linked to the polynucleotide encoding the foreign polypeptide. In some embodiments, the *Listeria* bacterium belongs to the species *Listeria monocytogenes*. In some embodiments, the codon-optimization of the polynucleotide enhances expression in and/or of the polypeptide from a *Listeria* bacterium of the polypeptide (relative to the non-codon-optimized sequence). In some embodiments, the foreign polypeptide comprises an antigen. In some embodiments, the foreign polypeptide is an antigen, which, in some instances, may be a non-bacterial antigen. For instance, the antigen is, in some embodiments a tumor-associated antigen or is derived from such a tumor-associated antigen. For instance, in some embodiments, the polypeptide is K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA, or is derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. In some embodiments, the antigen is mesothelin, or is an antigenic fragment or antigenic variant of mesothelin. In some other embodiments, the antigen is NY-ESO-1, or is an antigenic fragment or antigenic variant of NY-ESO-1. In some other embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, the expression cassette further comprises a polynucleotide encoding a signal peptide which is operably linked to the promoter and in the same translational frame as the foreign polypeptide so that the expression cassette encodes a fusion protein comprising the signal peptide and the foreign polypeptide. In some embodiments, the polynucleotide encoding the signal peptide (which may or may not be native to *Listeria*) is codon-optimized for expression in *Listeria monocytogenes*. Pharmaceutical compositions, immunogenic compositions, and vaccines comprising the recombinant *Listeria* bacteria are also provided. The invention further provides a method of inducing an immune response in host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium. In addition, the invention provides methods of using the recombinant *Listeria* bacteria to induce an immune response and/or prevent or treat a condition (e.g., a disease). The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the foreign polypeptide comprises the antigen is also provided.

[0104] In a further aspect, the invention provides a recombinant *Listeria* bacterium (e.g., *Listeria monocytogenes*) comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-*Listeria* signal peptide; and

(b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-Listerial signal peptide and the polypeptide. In some embodiments, the recombinant nucleic acid molecule is positioned in an expression cassette that further comprises a promoter operably linked to both the first and second polynucleotides. Thus, in some embodiments the recombinant *Listeria* bacterium comprises an expression cassette which comprises the recombinant nucleic acid molecule, wherein the expression cassette further comprises a promoter operably linked to both the first and second polynucleotides of the recombinant nucleic acid molecule. In some embodiments, the expression cassette is a polycistronic expression cassette (e.g., a bicistronic expression cassette). In some embodiments, the first polynucleotide, the second polynucleotide, or both the first and second polynucleotide are codon-optimized for expression in *Listeria* (e.g., *Listeria monocytogenes*). In some embodiments, the codon-optimization of the first and/or second polynucleotide enhances expression of the fusion protein in and/or secretion of the fusion protein from the bacterium (relative to the non-codon-optimized sequence). In some embodiments, the first and second polynucleotides are heterologous to each other. In some embodiments, the polypeptide encoded by the second polynucleotide and the signal peptide are heterologous to each other. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the *Listeria* bacterium (i.e., heterologous to the *Listeria* bacterium). In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen (e.g., a non-Listerial antigen). The polypeptide encoded by the second polynucleotide is, in some embodiments, an antigen. In some embodiments, the antigen is a tumor-associated antigen or is derived from a tumor-associated antigen. In some embodiments, the antigen is selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or is derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA. For instance, in some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant thereof. In some embodiments, the antigen is human mesothelin. In some embodiments, the antigen is human mesothelin deleted of its signal peptide and GPI linker domain. In some alternative embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, the signal peptide is bacterial. In some embodiments, the signal peptide is derived from an intracellular bacterium. In some embodiments, the signal peptide is derived from a gram positive bacterium. In some embodiments, the signal peptide is from a bacterium belonging to the genus *Bacillus*, *Staphylococcus*, or *Lactococcus* (e.g., *Bacillus anthracis*, *Bacillus subtilis*, *Staphylococcus aureus*, or *Lactococcus lactis*). In some embodiments, the signal peptide is a secA1 signal peptide (e.g., Usp45 signal peptide from *Lactococcus lactis* or Protective Antigen signal peptide from *Bacillus anthracis*). In some embodiments, the signal peptide is a secA2 signal peptide. In some embodiments the signal peptide is a Tat signal peptide (e.g., PhoD signal peptide from *B. sub-*

tilis). In some embodiments, the *Listeria* bacterium is attenuated. For instance, in some embodiments, the *Listeria* are attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the recombinant *Listeria* bacterium is deficient with respect to ActA, Internalin B, or both Act A and Internalin B (e.g., an Δ actA Δ inlB double deletion mutant). In some embodiments, the recombinant *Listeria* bacterium is deleted in functional ActA, Internalin B, or both Act A and Internalin B. In some embodiments, the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound (e.g., a psoralen compound). The invention also provides a pharmaceutical composition comprising the recombinant *Listeria* bacterium and a pharmaceutically acceptable carrier. The invention further provides an immunogenic composition comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is an antigen. The invention also provides a vaccine comprising the recombinant *Listeria* bacterium. Methods of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium, wherein the polypeptide encoded by the second polynucleotide is (or comprises) an antigen are also provided. Also provided are methods of preventing or treating a condition (e.g., a disease such as cancer or an infectious disease) in a host comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0105] In still another aspect, the invention provides a recombinant *Listeria* bacterium (for instance, from the species *Listeria monocytogenes*) comprising an expression cassette which comprises a first polynucleotide encoding a non-Listerial signal peptide, a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide, and a promoter operably linked to both the first and second polynucleotides. The expression cassette encodes a fusion protein comprising both the non-Listerial signal peptide and the polypeptide. In some embodiments, the *Listeria* bacterium is attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the first polynucleotide, the second polynucleotide, or both the first and second polynucleotides are codon-optimized for expression in *Listeria*. In some embodiments, the codon-optimization of the first and/or second polynucleotide enhances expression in and/or secretion from the bacterium of the encoded fusion protein (relative to the non-codon-optimized sequence). In some embodiments, the first polynucleotide and/or second polynucleotide is codon-optimized for expression in *Listeria monocytogenes*. In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen. In some embodiments, the polypeptide encoded by the second polynucleotide is an antigen, which, in some instances, may be a non-bacterial antigen. For instance, the antigen is, in some embodiments a tumor-associated antigen or is derived from such a tumor-associated antigen. For instance, in some embodiments, the antigen is K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17,

PAGE-4, TARP, or CEA, or is derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. For instance, in some embodiments, the antigen is mesothelin, or is an antigenic fragment or antigenic variant of mesothelin. In some other embodiments, the antigen is NY-ESO-1, or an antigenic fragment or antigenic variant of NY-ESO-1. In some embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In preferred embodiments, the signal peptide is bacterial. In some embodiments, the signal peptide is from a bacterium belonging to the genus *Bacillus*, *Staphylococcus*, or *Lactococcus*. For instance, in some embodiments, the signal peptide is from *Bacillus anthracis*, *Bacillus subtilis*, *Staphylococcus aureus*, or *Lactococcus lactis*. In some embodiments, the signal peptide is a secA1 signal peptide, such as a Usp45 signal peptide from *Lactococcus lactis* or a Protective Antigen signal peptide from *Bacillus anthracis*. In some embodiments, the signal peptide is a secA2 signal peptide. In still further embodiments, the signal peptide is a Tat signal peptide, such as a *B. subtilis* Tat signal peptide (e.g., PhoI). Pharmaceutical compositions, immunogenic compositions, and vaccines comprising the recombinant *Listeria* bacteria described herein are also provided. In addition, the invention provides methods of using the recombinant *Listeria* bacteria to induce an immune response and/or to prevent or treat a condition such as a disease. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0106] The invention further provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof; and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the polypeptide encoded by the second polynucleotide and the autolysin, or catalytically active fragment or catalytically active variant thereof, wherein, in the protein chimera, the polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof, or is positioned within the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the first polynucleotide encodes a bacterial autolysin. In some embodiments, the protein chimera is catalytically active as an autolysin. In some embodiments, the bacterial autolysin is from an intracellular bacterium (e.g., *Listeria*). In some embodiments, the bacterial autolysin is a Listerial autolysin. In some embodiments, the second polynucleotide encoding the polypeptide is positioned within the first polynucleotide encoding the autolysin, or catalytically active fragment or catalytically active variant thereof, and the recombinant nucleic acid molecule encodes a protein chimera in which the polypeptide is positioned within the autolysin, or catalytically active fragment or catalytically active variant thereof (i.e., the polypeptide is embedded within the autolysin or catalytically active fragment or variant). In some alternative embodiments, the second polynucleotide is positioned outside of the first polynucleotide encoding the autolysin, or catalytically active fragment or catalytically active variant thereof, and the recombinant

nucleic acid molecule encodes a protein chimera in which the polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the polypeptide is heterologous to the autolysin. In some embodiments, the first polynucleotide and the second polynucleotide are heterologous to each other. In some embodiments, the recombinant nucleic acid molecule further comprises (c) a third polynucleotide encoding a signal peptide in the same translational reading frame as the first and second polynucleotides, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the second polynucleotide, and the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the signal peptide is a secA2 signal peptide (such as p60). In some embodiments, the signal peptide is the signal peptide associated with the autolysin in nature (e.g., the signal peptide is p60 and the autolysin is p60). In some embodiments, the autolysin is a secA2-dependent autolysin. In some embodiments, the autolysin is a peptidoglycan hydrolase (e.g., N-acetylmuramidase or p60). In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen. In some embodiments, the polypeptide is an antigen (e.g., a tumor-associated antigen, an antigen derived from a tumor-associated antigen, an infectious disease antigen, or an antigen derived from an infectious disease antigen. In some embodiments, the antigen is selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA. For instance, in some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant thereof. In some embodiments, the antigen is human mesothelin. In some embodiments, the antigen is human mesothelin deleted of its signal peptide and GPI anchor. The invention also provides an expression cassette comprising the recombinant nucleic acid molecule, further comprising a promoter operably linked to the first and second polynucleotides of the recombinant nucleic acid molecule, as well as an expression vector comprising the expression cassette. The invention further provides a recombinant bacterium comprising the recombinant nucleic acid molecule. In some embodiments, the recombinant bacterium is an intracellular bacterium, such as a *Listeria* bacterium (e.g., *Listeria monocytogenes*). In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the recombinant bacterium. A pharmaceutical composition comprising (a) the recombinant bacterium, and (b) a pharmaceutically acceptable carrier is also provided. In addition, an immunogenic composition comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is an antigen, is also provided. Also provided is a vaccine comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is an antigen. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium, wherein the polypeptide encoded by the second polynucleotide is (or comprises) the antigen. A method of preventing or treating

a condition in a host comprising administering to the host an effective amount of a composition comprising the recombinant bacterium is also provided. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the second polynucleotide comprises the antigen is also provided.

[0107] In yet another aspect, the invention provides a recombinant *Listeria* bacterium comprising a polycistronic expression cassette, wherein the polycistronic expression cassette encodes at least two discrete non-*Listerial* polypeptides. For instance, in some embodiments, the expression cassette comprises a first polynucleotide encoding the first non-*Listerial* polypeptide, a second polynucleotide encoding the second non-*Listerial* polypeptide, and a promoter operably linked to the first and second polynucleotides. In some embodiments, the expression cassette further comprises an intergenic sequence between the first and second polynucleotides. In some embodiments, the polycistronic expression cassette is a bicistronic expression cassette which encodes two discrete non-*Listerial* polypeptides. In some embodiments, the recombinant *Listeria* bacterium belongs to the species *Listeria monocytogenes*. In some embodiments, at least one of the non-*Listerial* polypeptides encoded by the polycistronic expression cassette comprises an antigen. In some embodiments, at least two of the non-*Listerial* polypeptides each comprise fragments of the same antigen. In some embodiments, the antigen is a tumor-associated antigen or is derived from a tumor-associated antigen. For instance, in some embodiments, the antigen is an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or is derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA. In some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant thereof. In some embodiments, the antigen is human mesothelin. In some embodiments, the antigen is human mesothelin deleted of its signal peptide and GPI anchor. In some embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In some embodiments, at least one of the non-*Listerial* polypeptides encoded by the polycistronic expression cassette comprises a signal peptide (either a *Listerial* signal peptide or a non-*Listerial* signal peptide). In some embodiments, the signal peptide is a secA1 signal peptide. In some embodiments, the signal peptide is a secA2 signal peptide. In other embodiments, the signal peptide is a Tat signal peptide. In some embodiments, the expression cassette comprises a polynucleotide encoding the signal peptide, wherein the polynucleotide encoding the signal peptide is codon-optimized for expression in *Listeria*. The invention also provides a pharmaceutical composition comprising: (a) the recombinant *Listeria* bacterium, and (b) a pharmaceutically acceptable carrier. Also provided is an immunogenic composition comprising the recombinant *Listeria* bacterium. Also provided is a vaccine comprising the recombinant *Listeria* bacterium. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium is also provided wherein at least one of the non-*Listerial* polypeptides

comprises an antigen. A method of preventing or treating a condition in a host comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium is also provided. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein at least one of the non-*Listerial* polypeptides encoded by the polycistronic expression cassette comprises the antigen is also provided.

[0108] In other aspects, the invention provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide, (b) a second polynucleotide encoding a secreted protein, or a fragment thereof, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a third polynucleotide encoding a polypeptide heterologous to the secreted protein, or fragment thereof, wherein the third polynucleotide is in the same translational reading frame as the first and second polynucleotides, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the third polynucleotide, and the secreted protein, or fragment thereof, and wherein the polypeptide encoded by the third polynucleotide is fused to the secreted protein, or fragment thereof, or is positioned within the secreted protein, or fragment thereof, in the protein chimera. In some embodiments, the secreted protein is a naturally secreted protein (i.e., a protein that is secreted from its native cell). In some embodiments, the third polynucleotide is positioned within the second polynucleotide in the recombinant nucleic acid molecule, and the polypeptide encoded by the third polynucleotide is positioned with the secreted protein, or fragment thereof, in the protein chimera encoded by the recombinant nucleic acid molecule. In some embodiments, the third polynucleotide is positioned outside of the second polynucleotide in the recombinant nucleic acid molecule and the polypeptide encoded by the third polynucleotide is fused to the secreted protein or fragment thereof, in the protein chimera. An expression cassette comprising the recombinant nucleic acid molecule and further comprising a promoter operably linked to the first, second, and third polynucleotides of the recombinant nucleic acid molecule is also provided. In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen. In some embodiments, the polypeptide encoded by the second polynucleotide is an antigen. For instance, in some embodiments, the antigen is a tumor-associated antigen or is derived from a tumor-associated antigen. (e.g., an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or is derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA). In some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant thereof. For instance, in some embodiments, the antigen is human mesothelin or is human mesothelin deleted of its signal peptide and GPI anchor. In alternative embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. An expression vector comprising the expression cassette is also provided. Recombinant bacteria comprising the recombinant nucleic acid molecules are also provided. A recombi-

nant *Listeria* bacterium (e.g., *Listeria monocytogenes*) is also provided and in some embodiments, the polypeptide encoded by the third nucleotide is foreign to the *Listeria* bacterium. The invention also provides an immunogenic composition comprising the recombinant bacterium, wherein the polypeptide encoded by the third polynucleotide is an antigen. Also provided is a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium, wherein the polypeptide encoded by the third polynucleotide is (or comprises) an antigen. Pharmaceutical compositions and vaccines, comprising the bacteria are also provided, as are methods of using the recombinant bacteria or compositions comprising the bacteria to prevent or treat a condition in a host. The use of the bacterium in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein the polypeptide encoded by the third polynucleotide comprises the antigen is also provided.

[0109] In further aspects, the invention provides improved methods of expressing and secreting heterologous proteins in host bacteria. The invention also provides methods of improving expression and secretion of heterologous proteins in bacteria. The invention further provides methods of making the recombinant nucleic acid molecule, expression cassettes, expression vectors, and recombinant bacteria described herein.

[0110] The invention also provides a variety of polynucleotides useful in optimizing expression of heterologous polynucleotides in bacteria such as *Listeria*.

[0111] It will be understood that embodiments set forth in a Markush group, Markush claim, or by way of "or language," encompass each separate embodiment, any combination of each of separate embodiments, as well as an invention consisting of or comprising all of each of the separate embodiments, unless dictated otherwise explicitly or by the context.

[0112] Further descriptions of the aspects and embodiments described above as well as additional embodiments and aspects of the invention are provided below.

[0113] II. Recombinant Nucleic Acid Molecules

[0114] The invention provides a variety of polynucleotides useful for expression of polynucleotides, such as heterologous polynucleotides, in bacteria such as *Listeria*. For instance, recombinant nucleic acid molecules comprising novel combinations of sequences encoding signal peptides (or polypeptides comprising signal peptides) with coding sequences of polypeptides such as heterologous antigens are provided. Recombinant nucleic acid molecules comprising codon-optimized polynucleotide sequences are provided. In some embodiments, these recombinant nucleic acid molecules are heterologous in that they comprise polynucleotides (i.e., polynucleotide sequences) which are not naturally found in combination with each other as part of the same nucleic acid molecule. In some embodiments, the recombinant nucleic acid molecules are isolated. In some embodiments, the recombinant nucleic acid molecules are positioned within the sequences of expression cassettes, expression vectors, plasmid DNA within bacteria, and/or even the genomic DNA of bacteria (following insertion). In some embodiments, the recombinant nucleic acid molecules

provide enhanced expression and/or secretion of the polypeptide (e.g., a heterologous polypeptide) within a bacterium.

[0115] In some embodiments, the recombinant nucleic acid molecule is DNA. In some embodiments, the recombinant nucleic acid molecule is RNA. In some embodiments, the recombinant nucleic acid is single-stranded. In other embodiments, the recombinant nucleic acid is double-stranded.

[0116] In some embodiments, the recombinant nucleic acid molecules described herein encode a fusion protein such as fusion protein comprising a signal peptide and another polypeptide, such as a polypeptide heterologous to the signal peptide. In some embodiments, the signal peptide is a bacterial signal peptide. It is understood that the recited polypeptide components of a fusion protein may, but need not necessarily be, directly fused to each other. The polypeptide components of a fusion protein, may in some embodiments be separated on the polypeptide sequence by one or more intervening amino acid sequences. In some embodiments the other polypeptide is non-bacterial, for instance, mammalian or viral.

[0117] For instance, in one aspect, the invention provides a recombinant nucleic acid molecule, comprising: (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium; and (b) a second polynucleotide encoding a polypeptide (e.g., an antigen), wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In additional embodiments, the second polynucleotide (the polynucleotide encoding the polypeptide, such as an antigen) is also codon-optimized for expression in a bacterium. The bacterium for which the first and/or second polynucleotide is codon-optimized should be the bacterium of a type in which the recombinant nucleic acid molecule is intended to be placed.

[0118] In another aspect, the invention provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the second polynucleotide is heterologous to the first polynucleotide. In some embodiments, the polypeptide is heterologous to the bacterium to which the signal peptide is native (i.e., foreign to the bacterium). In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide, foreign to the bacterium, or both. In some embodiments, the bacterium from which the signal peptide is derived is an intracellular bacterium. In some embodiments, the bacterium is selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria and *E. coli*. In some embodiments, the signal peptide is native to a *Listeria* bacterium. In some

embodiments, the signal peptide is native to a *Listeria* bacterium belonging to the species *Listeria monocytogenes*. In some embodiments, the second polynucleotide is codon-optimized for expression in the bacterium.

[0119] In another aspect, the invention provides a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a *Listeria* bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the signal peptide is native to the *Listeria* bacterium. In some other embodiments, the signal peptide is foreign to the *Listeria* bacterium. In some embodiments, the signal peptide is heterologous to the polypeptide encoded by the second polynucleotide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the *Listeria* bacterium. In some embodiments, the *Listeria* bacterium belongs to the species *Listeria monocytogenes*.

[0120] The invention also provides a recombinant nucleic acid molecule comprising a polynucleotide encoding a polypeptide foreign to a *Listeria* bacterium (e.g., a cancer or non-*Listeria* infectious disease antigen), wherein the polynucleotide encoding the foreign polypeptide is codon-optimized for expression in the *Listeria* bacterium.

[0121] In another aspect, the invention provides a recombinant nucleic acid molecule, comprising: (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide, and (b) a second polynucleotide encoding a polypeptide, such as an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the non-secA1 bacterial signal peptide is a secA2 signal peptide or a Tat signal peptide. In some embodiments, the first polynucleotide encoding the non-secA1 signal peptide is codon-optimized for expression in the bacteria in which the recombinant nucleic acid molecule is intended to be placed (e.g., *Listeria*). In some embodiments, the second polynucleotide encoding a polypeptide, such as an antigen, is codon-optimized for expression in the bacteria in which the recombinant nucleic acid molecule is intended to be placed. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the bacterium in which the recombinant nucleic acid molecule is to be incorporated or has been incorporated. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the bacterium in which the recombinant nucleic acid molecule is to be incorporated or has been incorporated and the polypeptide encoded by the second polynucleotide is also heterologous to the signal peptide.

[0122] The invention further provides a recombinant nucleic acid molecule, comprising a first polynucleotide encoding a non-secA1 bacterial signal peptide, a second polynucleotide encoding a polypeptide (e.g., heterologous

protein and/or antigen), and a third polynucleotide encoding a SecA2 autolysin, or fragment thereof, in the same translational reading frame as the first and second polynucleotides, wherein the second polynucleotide is positioned within the third polynucleotide or between the first and third polynucleotides. In some embodiments, the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide, the polypeptide, and the autolysin. In some embodiments, the fragment of the autolysin is catalytically active as an autolysin. In some embodiments, the autolysin is from an intracellular bacterium. In some embodiments, the autolysin is a peptidoglycan hydrolase. In some embodiments, the bacterial autolysin is a *Listeria* autolysin. In some embodiments, the autolysin is p60. In some embodiments, the autolysin is N-acetylmuramidase.

[0123] The invention also provides a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-*Listeria* signal peptide; and (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-*Listeria* signal peptide and the polypeptide. In some embodiments, the non-*Listeria* signal peptide is heterologous to the polypeptide encoded by the second polynucleotide. In some embodiments, the first polynucleotide, the second polynucleotide, or both the first and second polynucleotides are codon-optimized for expression in a *Listeria* bacterium.

[0124] The invention also provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the polypeptide encoded by the second polynucleotide and the autolysin, or catalytically active fragment or catalytically active variant thereof, wherein, in the protein chimera, the polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof, or is positioned within the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the second polynucleotide is positioned within the first polynucleotide, and the recombinant nucleic acid molecule encodes a protein chimera in which the polypeptide encoded by the second polynucleotide is positioned within the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the second polynucleotide is positioned outside the second polynucleotide, and the recombinant nucleic acid molecule encodes a protein chimera in which the polypeptide encoded by the second polynucleotide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the first polynucleotide encodes an autolysin. In some embodiments, the recombinant nucleic acid molecule further comprises (c) a third polynucleotide encoding a signal peptide in the same translational reading frame as the first and second polynucleotides, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the second polynucleotide, and the autolysin, or catalytically active fragment or catalytically active variant thereof.

In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the autolysin. In some embodiments, the fragments of the autolysin are at least about 30, at least about 40, at least about 50, or at least about 100 amino acids in length. In some embodiments, the autolysin is from an intracellular bacterium. In some embodiments, the bacterial autolysin is a *Listeria* autolysin. Catalytically active variants of an autolysin include variants that differ from the original autolysin in one or more substitutions, deletions, additions, and/or insertions. In some embodiments, the autolysin is a peptidoglycan hydrolase. In some embodiments, the autolysin is p60. In some embodiments, the autolysin is N-acetylmuramidase.

[0125] Additional autolysins can be identified and characterized by zymography, a technique known to those skilled in the art (see, e.g., Lenz, et al. (2003) *Proc. Natl. Acad. Sci. USA* 100:12432-12437). Zymography can also be used to determine whether a given fragment and/or variant of an autolysin is catalytically active as an autolysin. The technique can also be used to assess whether or not a particular protein chimera is catalytically active as an autolysin.

[0126] In some embodiments, the catalytically active fragments and/or variants of the autolysin are at least about 10%, at least about 30%, at least about 50%, at least about 75%, at least about 90%, or at least about 95% as catalytically active as an autolysin as the native autolysin.

[0127] In some embodiments, the protein chimera is catalytically active as an autolysin. In some embodiments, the protein chimera is at least about 10%, at least about 30%, at least about 50%, at least about 75%, at least about 90%, or at least about 95% as catalytically active as an autolysin as the native autolysin.

[0128] Another option for heterologous protein expression is to utilize a protein "scaffold" into which a heterologous protein is functionally inserted "in-frame." In this composition, whole genes or components of the gene corresponding to, for example, MHC class I or MHC class II epitopes are inserted within and through a scaffold protein. The scaffold protein can be a highly expressed bacterial proteins (such as a *Listeria* protein, like LLO or p60), but in another embodiment can be a heterologous protein that is selected for its high expression, stability, secretion, and/or (lack of) immunogenicity. Representative examples of scaffold proteins are chicken ovalbumin, or other human proteins, such as β -globin or albumin.

[0129] The invention also provides a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide, (b) a second polynucleotide encoding a secreted protein, or a fragment thereof, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a third polynucleotide encoding a polypeptide heterologous to the secreted protein, or fragment thereof, wherein the third polynucleotide is in the same translational reading frame as the first and second polynucleotides, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide heterologous to the secreted protein, and the secreted protein, or fragment thereof, and wherein the polypeptide is fused to the secreted protein, or fragment thereof, or is positioned within the secreted protein, or fragment thereof, in the protein chimera. In some embodiments, the second polynucleotide encodes a secreted

protein. In some embodiments, the secreted protein is a protein that is secreted from its native cell. In some embodiments, the third polynucleotide is positioned within the second polynucleotide in the recombinant nucleic acid molecule, and the polypeptide encoded by the third polynucleotide is positioned with the secreted protein, or fragment thereof, in the protein chimera encoded by the recombinant nucleic acid molecule. In some embodiments, the third polynucleotide is positioned outside of the second polynucleotide in the nucleic acid molecule and the polypeptide encoded by the third polynucleotide is fused to the secreted protein or fragment thereof, in the protein chimera. In some embodiments, the secreted protein is ovalbumin. In some embodiments, a truncated form of ovalbumin is used. In some embodiments, the secreted protein is p60. In some embodiments, the secreted protein is N-acetylmuramidase. In some embodiments, the signal peptide is the signal peptide normally associated with the secreted protein. In some embodiments, the signal peptide is heterologous to the secreted protein. In some embodiments, the fragments of the secreted protein are at least about 30, at least about 40, at least about 50, or at least about 100 amino acids in length.

[0130] In some embodiments, the recombinant nucleic acid molecule, expression cassette, or expression vector comprises a coding sequence for a polypeptide that is foreign to the bacteria, embedded within part or a whole coding sequence of a protein that is highly expressed within the bacteria. In some embodiments, the highly expressed sequence is native to the bacteria in which the sequence is to be expressed. In other embodiments, the highly expressed sequence is not native to the bacteria in which it is to be expressed, but provides sufficient expression, nonetheless.

[0131] In another aspect, the invention provides a recombinant nucleic acid molecule, wherein the nucleic acid molecule encodes at least two discrete non-*Listeria* polypeptides. In some embodiments, the polynucleotides encoding the non-*Listeria* polypeptides are codon-optimized for expression in a *Listeria* bacterium.

[0132] Methods of preparing recombinant nucleic acid molecules, including those described above, are well known to those of ordinary skill in the art. For instance, recombinant nucleic acid molecules can be prepared by synthesizing long oligonucleotides on a DNA synthesizer which overlap with each other and then performing extension reaction and/or PCR to generate the desired quantity of double-stranded DNA. The double-stranded DNA can be cut with restriction enzymes and inserted into the desired expression or cloning vectors. Sequencing may be performed to verify that the correct sequence has been obtained. Also by way of non-limiting example, alternatively, one or more portions of the recombinant nucleic acid molecules may be obtained from plasmids containing the portions. PCR of the relevant portions of the plasmid and/or restriction enzyme excision of the relevant portions of the plasmid can be performed, followed by ligation and/or PCR to combine the relevant polynucleotides to generate the desired recombinant nucleic acid molecules. Such techniques are standard in the art. Standard cloning techniques may also be used to insert the recombinant nucleic acid sequence into a plasmid and replicate the recombinant nucleic acid within a host cell, such as bacteria. The recombinant nucleic acid can then be isolated from the host cell.

[0133] The invention also provides a method of using any of the recombinant nucleic acid molecules described herein to produce a recombinant bacterium (e.g. a recombinant *Listeria* bacterium). In some embodiments, the method of using a recombinant nucleic acid molecule described herein to make a recombinant bacterium comprises introducing the recombinant nucleic acid molecule into a bacterium. In some embodiments, the recombinant nucleic acid molecule is integrated into the genome of the bacterium. In some other embodiments, the recombinant nucleic acid molecule is on a plasmid which is incorporated within the bacterium. In some embodiments, incorporation of the recombinant nucleic acid molecule into the bacterium occurs by conjugation. The introduction into the bacterium can be effected by any of the standard techniques known in the art. For instance, incorporation of the recombinant nucleic acid molecule into the bacterium can occur by conjugation, transduction (transfection), or transformation.

[0134] III. Signal Peptides

[0135] In some embodiments, the recombinant nucleic acid molecules, expression cassettes, and/or vectors of the invention encode fusion proteins or protein chimeras which comprise signal peptides and are suitable for expression in and secretion from host cells such as bacteria. Thus, in some embodiments, the recombinant nucleic acid molecules, expression cassettes and/or vectors of the invention comprise polynucleotides encoding signal peptides.

[0136] The terms "signal peptide" and "signal sequence," are used interchangeably herein. In some embodiments, the signal peptide helps facilitate transportation of a polypeptide fused to the signal peptide across the cell membrane of a cell (e.g., a bacterial cell) so that the polypeptide is secreted from the cell. Accordingly, in some embodiments, the signal peptide is a "secretory signal peptide" or "secretory sequence". In some embodiments, the signal peptide is positioned at the N-terminal end of the polypeptide to be secreted.

[0137] In some embodiments, the sequence encoding the signal peptide in the recombinant nucleic acid molecule or expression cassette is positioned within the recombinant nucleic acid molecule or expression cassette such that the encoded signal peptide will effect secretion, of the polypeptide to which it is fused from the desired host cell (e.g., a bacterium). In some embodiments, in a recombinant nucleic acid molecule or an expression cassette, the polynucleotide encoding the signal peptide is positioned in frame (either directly or separated by intervening polynucleotides) at the 5' end of the polynucleotide that encodes the polypeptide to be secreted (e.g., a polypeptide comprising an antigen).

[0138] In some embodiments, the signal peptides that are a part of the fusion proteins and/or protein chimeras encoded by the recombinant nucleic acid molecules, expression cassettes and/or expression vectors, are heterologous to at least one other polypeptide sequence in the fusion protein and/or protein chimera. In some embodiments, the signal peptide encoded by the recombinant nucleic acid molecule, expression cassette and/or expression vector is heterologous (i.e., foreign) to the bacterium into which the recombinant nucleic acid molecule, expression cassette and/or expression vector is to be incorporated or has been incorporated. In some

embodiments, the signal peptide is native to the bacterium in which the recombinant nucleic acid molecule, expression cassette and/or expression vector is to be incorporated.

[0139] In some embodiments, the polynucleotide encoding the signal peptide is codon-optimized for expression in a bacterium (e.g., *Listeria* such as *Listeria monocytogenes*). In some embodiments, the polynucleotide that is codon-optimized for a particular bacterium is foreign to the bacterium. In other embodiments, the polynucleotide that is codon-optimized for a particular bacterium is native to that bacterium.

[0140] A large variety of signal peptides are known in the art. In addition, a variety of algorithms and software programs, such as the "SignalP" algorithms, which can be used to predict signal peptide sequences are available in the art. For instance, see: Antelmann et al., *Genome Res.*, 11:1484-502 (2001); Menne et al., *Bioinformatics*, 16:741-2 (2000); Nielsen et al., *Protein Eng.*, 10:1-6 (1997); Zhang et al., *Protein Sci.*, 13:2819-24 (2004); Bendtsen et al., *J. Mol. Biol.*, 340:783-95 (2004) (regarding SignalP 3.0); Hiller et al., *Nucleic Acids Res.*, 32:W375-9 (2004); Schneider et al., *Proteomics* 4:1571-80 (2004); Chou, *Curr. Protein Pept. Sci.*, 3:615-22 (2002); Shah et al., *Bioinformatics*, 19:1985-96 (2003); and Yuan et al., *Biochem. Biophys. Res. Commun.* 312:1278-83 (2003).

[0141] In some embodiments the signal peptide is prokaryotic. In some alternative embodiments, the signal peptide is eukaryotic. The use of eukaryotic signal peptides for expression of proteins in *Escherichia coli* for example, is described in Humphreys et al., *Protein Expression and Purification*, 20:252-264 (2000).

[0142] In some embodiments, the signal peptide is a bacterial signal peptide. In some embodiments, the signal peptide is a non-*Listeria* signal peptide. In some embodiments, the signal peptide is a *Listeria* signal peptide. In some embodiments the signal peptide is derived from a gram-positive bacterium. In some embodiments, the signal peptide is derived from an intracellular bacterium.

[0143] In some embodiments, the signal peptide (e.g., a non-secA1 bacterial signal peptide) used in a recombinant nucleic acid molecule, expression cassette, or expression vector is derived from *Listeria*. In some embodiments, this signal peptide is derived from *Listeria monocytogenes*. In some embodiments, the signal peptide is a signal peptide from *Listeria monocytogenes*. In some embodiments, the signal peptide is not derived from *Listeria*, but is instead derived from a bacterium other than a bacterium belonging to the genus *Listeria*. In some embodiments, the bacterial signal peptide is derived from a *Bacillus* bacterium. In some embodiments, the bacterial signal peptide is derived from *Bacillus subtilis*. In some embodiments, the bacterial signal peptide is derived from a bacterium belonging to the genus *Staphylococcus*. In some embodiments, the bacterial signal peptide is derived from a *Lactococcus* bacterium. In some embodiments, the bacterial signal peptide is derived from a *Bacillus*, *Staphylococcus*, or *Lactococcus* bacterium. In some embodiments, the bacterial signal peptide is a signal peptide from a *Bacillus*, *Staphylococcus*, or *Lactococcus*

bacterium. In some embodiments, the bacterial signal peptide is a signal peptide derived from *Bacillus anthracis*, *Bacillus subtilis*, *Staphylococcus aureus*, or *Lactococcus lactis*. In some embodiments, the bacterial signal peptide is a signal peptide from *Bacillus anthracis*. In some embodiments, the bacterial signal peptide is a signal peptide from *Bacillus subtilis*. In some embodiments, the bacterial signal peptide is a signal peptide from *Lactococcus lactis*. In some embodiments, the bacterial signal peptide is a signal peptide from *Staphylococcus aureus*.

[0144] In some embodiments of the polynucleotides described herein, the signal peptide that is derived from an organism, such as a bacterium, is identical to a naturally occurring signal peptide sequence obtained from the organism. In other embodiments, the signal peptide sequence encoded by the recombinant nucleic acid molecule, expression cassette, and/or expression vector is derived from a naturally occurring signal peptide sequence, i.e., a fragment and/or variant of a naturally occurring signal peptide sequence, wherein the fragment or variant still functions as a signal peptide. A variant includes polypeptides that differ from the original sequence by one or more substitutions, deletions, additions, and/or insertions. For instance, in some embodiments the signal peptide that is encoded by the polynucleotides contains one or more conservative mutations. Possible conservative amino acid changes are well known to those of ordinary skill in the art. See, e.g., Section IV of the Detailed Description, below, for additional information regarding conservative amino acid changes.

[0145] A signal peptide derived from another signal peptide (i.e., a fragment and/or variant of the other signal peptide) is preferably substantially equivalent to the original signal peptide. For instance, the ability of a signal peptide derived from another signal peptide to function as a signal peptide should be substantially unaffected by the variations (deletions, mutations, etc.) made to the original signal peptide sequence. In some embodiments, the derived signal peptide is at least about 70%, at least about 80%, at least about 90%, or at least about 95% able to function as a signal peptide as the native signal peptide sequence. In some embodiments, the signal peptide has at least about 70%, at least about 80%, at least about 90%, or at least about 95% identity in amino acid sequence to the original signal peptide. In some embodiments, the only alterations made in the sequence of the signal peptide are conservative amino acid substitutions. Fragments of signal peptides are preferably at least about 80% or at least about 90% of the length of the original signal peptides.

[0146] In some embodiments, the signal peptide encoded by a polynucleotide in the recombinant nucleic acid molecules, expression cassettes, or expression vectors is a secA1 signal peptide, a secA2 signal peptide, or a Twin-arginine translocation (Tat) signal peptide. In some embodiments, the signal peptide is a secA1 signal peptide. In some embodiments, the signal peptide is a non-secA1 signal peptide. In some embodiments, the signal peptide is a secA2 signal peptide. In some embodiments, the signal peptide is a twin-arginine translocation (Tat) signal peptide. In some embodiments, these secA1, secA2, or Tat signal peptides are derived from *Listeria*. In some embodiments, these secA1,

secA2, or Tat signal peptides are non-*Listeria*. For instance, in some embodiments, the secA1, secA2, and Tat signal peptides are derived from bacteria belonging to one of the following genera: *Bacillus*, *Staphylococcus*, or *Lactococcus*.

[0147] Bacteria utilize diverse pathways for protein secretion, including secA1, secA2, and Twin-Arg Translocation (Tat). Which pathway is utilized is largely determined by the type of signal sequence located at the N-terminal end of the pre-protein. The majority of secreted proteins utilize the Sec pathway, in which the protein translocates through the bacterial membrane-embedded proteinaceous Sec pore in an unfolded conformation. In contrast, the proteins utilizing the Tat pathway are secreted in a folded conformation. Nucleotide sequence encoding signal peptides corresponding to any of these protein secretion pathways can be fused genetically in-frame to a desired heterologous protein coding sequence. The signal peptides optimally contain a signal peptidase cleavage site at their carboxyl terminus for release of the authentic desired protein into the extra-cellular environment (Sharkov and Cai. 2002 *J. Biol. Chem.* 277:5796-5803; Nielsen et. al. 1997 *Protein Engineering* 10:1-6; and, www.cbs.dtu.dk/services/SignalP/).

[0148] The signal peptides used in the polynucleotides of the invention can be derived not only from diverse secretion pathways, but also from diverse bacterial genera. Signal peptides generally have a common structural organization, having a charged N-terminus (N-domain), a hydrophobic core region (H-domain) and a more polar C-terminal region (C-domain), however, they do not show sequence conservation. In some embodiments, the C-domain of the signal peptide carries a type I signal peptidase (SPase I) cleavage site, having the consensus sequence A-X-A, at positions -1 and -3 relative to the cleavage site. Proteins secreted via the sec pathway have signal peptides that average 28 residues. The secA2 protein secretion pathway was first discovered in *Listeria monocytogenes*; mutants in the secA2 paralogue are characterized by a rough colony phenotype on agar media, and an attenuated virulence phenotype in mice (Lenz and Portnoy, 2002 *Mol. Microbiol.* 45:1043-1056; and, Lenz et. al 2003 *PNAS* 100:12432-12437). Signal peptides related to proteins secreted by the Tat pathway have a tripartite organization similar to Sec signal peptides, but are characterized by having an RR-motif (R—R—X—#, where # is a hydrophobic residue), located at the N-domain/H-domain boundary. Bacterial Tat signal peptides average 14 amino acids longer than sec signal peptides. The *Bacillus subtilis* secretome may contain as many as 69 putative proteins that utilize the Tat secretion pathway, 14 of which contain a SPase I cleavage site (Jongbloed et. al. 2002 *J. Biol. Chem.* 277:44068-44078; Thalsma et. al., 2000 *Microbiol. Mol. Biol. Rev.* 64:515-547).

[0149] Shown in Table 1 below are non-limiting examples of signal peptides that can be used in fusion compositions (including protein chimera compositions) with a selected other polypeptide such as a heterologous polypeptide, resulting in secretion from the bacterium of the encoded protein.

TABLE 1

Some exemplary signal peptides				
Secretion Pathway	Signal Peptide Amino Acid Sequence (NH ₂ -CO ₂)	Signal peptidase Site (cleavage site represented by ')	Gene	Genus/species
secA1	MKKIMLVFITLLVSLPIAQQ	TEA'KD	hly (LLO)	<i>Listeria monocytogenes</i>
	TEAKD (SEQ ID NO: 45)	(SEQ ID NO: 54)		
	MKKKIIISAILMS'I'VILSAAAP	VYA'DI'	Usp45	<i>Lactococcus lactis</i>
	LSGVYADT (SEQ ID NO: 46)	(SEQ ID NO: 55)		
	MKRRKVLIPLMALSTILVSS	IQA'EV	pag	<i>Bacillus anthracis</i>
secA2	TGNLEVIQAEV (SEQ ID NO: 47)	(SEQ ID NO: 56)	(Protective Antigen)	
	MNMKKATIAATAGIAVTAF	ASA'ST	iap	<i>Listeria monocytogenes</i>
	AAPTIASAST' (SEQ ID NO: 48)	(SEQ ID NO: 57)	invasion-associated protein p60	
	MOKTRKERILEALQEEKKN	VSA'DE	NamA	<i>Listeria monocytogenes</i>
	KSKKFKFTGATIAGVTAIAT	(SEQ ID NO: 58)	lmo2691	
Tat	SITVPGIFIVISADE (SEQ ID NO: 49)	(autolysin)		
	MKKLKMASCALVAGLMFS	* BA_0281		<i>Bacillus anthracis</i>
	GLTPNAFAED (SEQ ID NO: 50)	(SEQ ID NO: 59)	(NLP/P60 Family)	
	MAKKFNYKLP'SMVALTLVG	VQA'AE	* atl	<i>Staphylococcus aureus</i>
	SAVTAHQVQAAE (SEQ ID NO: 51)	(SEQ ID NO: 60)	(autolysin)	
Tat	MTDKKSENQTEKTETKENK	DKA'LT	lmo0367	<i>Listeria monocytogenes</i>
	GMTTRREMLKLSAVAGTGIA	(SEQ ID NO: 61)		
	VGATGLGTILNVVDQVDKA			
	LT' (SEQ ID NO: 52)			
	MAYDSRFDEWVQKLKEESF	VGA'FG	PhoD	<i>Bacillus subtilis</i>
	QNNTFDRRKFIQAGAGKIAGL	(SEQ ID NO: 62)	(alkaline phosphatase)	
	SLGLITAAQSVGAFG (SEQ ID NO: 53)			

* Bacterial autolysins secreted by sec pathway (not determined whether secA1 or secA2).

[0150] Accordingly, in some embodiments, the sequence that encodes the signal peptide encodes a secA1 signal peptide. An example of a secA1 signal peptide is the Listeriolysin O (LLO) signal peptide from *Listeria monocytogenes*. In some embodiments, the recombinant nucleic acid molecule or expression cassette comprising a polynucleotide encoding an LLO signal peptide further comprises a polynucleotide sequence encoding the LLO PEST sequence. Other examples of secA1 signal peptides suitable for use in the present invention include the signal peptides from the Usp45 gene in *Lactococcus lactis* (see Table 1, above, and Example 12 below) and Pag (Protective Antigen) gene from *Bacillus anthracis*. Thus, in some embodiments, the signal peptide is a protective antigen signal peptide from *Bacillus anthracis*. In some other embodiments, the signal peptide is a secA1 signal peptide other than the protective antigen signal peptide from *Bacillus anthracis*. Another example of a secA1 signal peptide is the SpsB signal peptide from *Staphylococcus aureus* (Sharkov et al., *J. of Biological Chemistry*, 277: 5796-5803 (2002)).

[0151] In some alternative embodiments, the heterologous coding sequences are genetically fused with signal peptides that are recognized by the secA2 pathway protein secretion complex. An auxiliary SecA paralog (SecA2) has been identified in nine Gram-positive bacteria that cause severe or lethal infections of humans. SecA2 is required for secretion of a subset of the exported proteomes (secretomes) of *Listeria*, *Mycobacteria*, and *Streptococci* (Braunstein et al., *Mol. Microbiol.* 48:453-64 (2003); Bensing et al., *Mol. Microbiol.*, 44:1081-94 (2002); Lenz et al., *Mol. Microbiol.*, 45:1043-1056 (2002); and Braunstein et al., *J. Bacteriology*, 183:6979-6990 (2001)). The *Listeria monocytogenes* SecA2 was identified through its association with bacterial smooth-

rough variation, and mutations in secA2 reduced virulence of *L. monocytogenes* and *Mycobacterium tuberculosis*.

[0152] For example, the *Listeria* protein p60 is a peptidoglycan autolysin that is secreted by the secA2 pathway. As an example, the secA2 signal peptide and signal peptidase cleavage site from p60 can be linked genetically with the amino terminus of a desired protein (e.g. antigen)-encoding gene. In one embodiment, the pre-protein comprised of the secA2 signal peptide and signal peptidase-antigen fusion is translated from an expression cassette within a bacterium, transported through the Gram-positive cell wall, in which the authentic heterologous protein is released into the extracellular milieu.

[0153] Alternatively, a heterologous sequence can be incorporated "in-frame" within p60, such that the heterologous protein is secreted in the form of a chimeric p60-heterologous protein. Insertion of the heterologous protein coding sequence in-frame into p60 can occur, for example, at the junction between the signal peptidase cleavage site and the mature p60 protein. In this embodiment, the chimeric protein retains the appropriate secA2 secretion signals, and also its autolysin activity, meaning that the heterologous protein is secreted as a gratuitous passenger of p60. In-frame incorporation of the heterologous antigen into p60 can be engineered at any point within p60 that retains both the secretion and autolysin activities of the p60 protein. An example of a partial expression cassette suitable for insertion of the desired antigen or other heterologous polypeptide coding sequence is described in Example 13, below.

[0154] In some embodiments, the fusion protein encoded by the recombinant nucleic acid molecule is a chimera comprising a bacterial protein having a particular desirable property (in addition to the desired heterologous protein

such as an antigen). In some embodiments the chimera comprises a hydrolase. In some embodiments, the recombinant nucleic acid molecule encodes a p60 chimera comprising the endopeptidase p60, a peptidoglycan hydrolase that degrades the bacterial cell wall. In some embodiments, the fusion protein encoded by the recombinant nucleic acid molecule comprises a *L. monocytogenes* hydrolase, for example, p60 (see, e.g., Genbank accession no. NP_464110) or N-acetylmuramidase (NamA) (Genbank accession no. NP_466213), both of which are secA2 dependent secreted proteins that degrade the cell wall. Such particular protein chimera compositions take advantage of not only molecular chaperones required for secretion of bacterial proteins, but also of the activity of the bacterial protein that can facilitate its secretion. Particular protein chimeras comprised of precise placement of a heterologous protein encoding sequence with a *L. monocytogenes* hydrolase result in the efficient expression and secretion of the heterologous protein. (See, e.g., the specific example, Example 29, below.) Accordingly, in some embodiments, the signal peptide encoded by the recombinant nucleic acid molecule as part of a fusion protein is p60 signal peptide. In some embodiments, the signal peptide encoded by the recombinant nucleic acid molecule as part of a fusion protein is a NamA signal peptide.

[0155] In some embodiments, the recombinant nucleic acid molecule comprises a third polynucleotide sequence encoding p60 protein, or a fragment thereof, in the same translational reading frame as both the first polynucleotide encoding the p60 signal peptide and the second polynucleotide encoding the other polypeptide (e.g., antigen). The recombinant nucleic acid molecule then encodes a fusion protein comprising the signal peptide, the polypeptide encoded by the second polynucleotide (e.g., an antigen), and the p60 protein, or a fragment thereof. In such embodiments, the second polynucleotide is preferably positioned either within the third polynucleotide or between the first and third polynucleotides.

[0156] In some embodiments, the secA2 signal peptide is a secA2 signal peptide derived from *Listeria*. For instance, in some embodiments, the signal peptide is a secA2 signal peptide such as the p60 signal peptide or the N-acetylmuramidase (NamA) signal peptide from *L. monocytogenes*. In addition, other *L. monocytogenes* proteins have been identified as not being secreted in the absence of secA2 (Lenz et al., Mol. Microbiology 45:1043-1056 (2002)) and polynucleotides encoding the signal peptides from these proteins can be used in some embodiments. Additionally, secA2 signal peptides from bacteria other than *Listeria* can be utilized for expression and secretion of heterologous proteins from recombinant *Listeria* or other bacteria. For instance, as an illustrative but non-limiting example, secA2 signal peptides from *B. anthracis* can be used in the recombinant nucleic acid molecules and/or expression cassettes. In other embodiments, a secA2 signal peptide from *S. aureus* is used. See Table 1. Proteins secreted via the SecA2 pathway in other bacteria have also been identified (see, e.g., Braunstein et al., Mol. Microbiol., 48:453-64 (2003) and Bensing et al., Mol. Microbiol. 44:1081-94 (2002)).

[0157] Additional proteins secreted via the secA2 pathway can be identified. SecA2 homologues have been identified in a number of bacterial species (see, e.g., Lenz et al., Mol. Microbiology 45:1043-1056 (2002) and Braunstein et al., J.

Bacteriology, 183:6979-6990 (2001)). Additional secA2 homologues can be identified by further sequence comparison using techniques known to those skilled in the art. Once a homologue is identified, the homologue can be deleted from the bacterial organism to generate a Δ secA2 mutant. The supernatant proteins of the wild-type and mutant bacterial cultures can be TCA-precipitated and analyzed by any of the proteomics techniques known in the art to determine which proteins are secreted by the wild-type bacteria, but not the Δ secA2 mutant. For instance, the secreted proteins can be analyzed via SDS-PAGE and silver staining. The resulting bands can be compared to identify those proteins for which secretion did not occur in the absence of the SecA2. (See, e.g., Lenz et al., Mol. Microbiology 45:1043-1056 (2002)). The N-terminal sequences of these proteins can then be analyzed (e.g., with an algorithm to predict the signal peptide cleavage site) to determine the secA2 signal peptide sequence used by that protein. N-terminal sequencing by automated Edman degradation can also be performed to identify the sequence of the signal peptide.

[0158] In alternative embodiments, the polynucleotides encode polypeptides (e.g., heterologous polypeptide sequences) that are genetically fused with signal peptides that are recognized by the Tat pathway protein secretion complex. The Tat secretion pathway is utilized by bacteria, including *Listeria* spp., for secretion of proteins that are folded within the bacterium. For example, the *Listeria innocua* protein YwbN has a putative Tat motif at its amino terminus and thus uses the Tat pathway for secretion (Genbank Accession No. NP_469731 [gi16799463]ref[NP_469731.1] conserved hypothetical protein similar to *B. subtilis* YwbN protein (*Listeria innocua*)), incorporated by reference herein). Another protein containing a Tat signal peptide is the YwbN protein from *Listeria monocytogenes* strain EGD(c) (Genbank Accession No. NP_463897 [gi16802412]ref[NP_463897.1] conserved hypothetical protein similar to *B. subtilis* YwbN protein (*Listeria monocytogenes* EGD (c))). As an example, the YwbN signal peptide and signal peptidase cleavage site from YwbN can be linked genetically with the amino terminus of a desired protein (e.g. antigen)-encoding gene. In this composition, the pre-protein comprised of the Tat signal peptide and signal peptidase-antigen fusion will be translated from an expression cassette within the bacterium, transported through the Gram-positive cell wall, in which the authentic heterologous protein is released into the extracellular milieu. Another protein predicted to be secreted from *Listeria innocua* via the Tat pathway is 3-oxoacyl-acyl carrier protein synthase (Genbank Accession No. NP_471636 [gi16801368]ref[NP_471636.1] similar to 3 (oxoacyl(acyl-carrier protein synthase (*Listeria innocua*))). Polynucleotides encoding signal sequences from any of these proteins predicted to be secreted from *Listeria* via the Tat secretory pathway may be used in the polynucleotides, expression cassettes, and/or expression vectors described herein.

[0159] Tat signal sequences from other bacteria can also be used as signal peptides, including, but not restricted to, phoD from *B. subtilis*. Examples of Tat signal peptides from *Bacillus subtilis*, such as phoD, are described in Jongbloed et al., J. of Biological Chemistry, 277:44068-44078 (2002); Jongbloed et al., J. of Biological Chemistry, 275:41350-41357 (2000), Pop et al., J. of Biological Chemistry, 277:3268-3273 (2002); van Dijk et al., J. of Biotechnology, 98:243-254 (2002); and Tjalsma et al., Microbiology and

Molecular Biology Reviews, 64: 515-547 (2000), all of which are incorporated by reference herein in their entirety. Other proteins identified in *B. subtilis* that have been predicted to be secreted by the Tat pathway include those sequences having the following Genbank/Embl Accession Nos.: CAB15017 [gi|2635523|emb|CAB15017.1] similar to two (component sensor histidine kinase (YtsA) (*Bacillus subtilis*)); CAB12056 [gi|2632548|emb|CAB12056.1] phosphodiesterase/alkaline phosphatase D (*Bacillus subtilis*); CAB12081 [gi|2632573|emb|CAB12081.1] similar to hypothetical proteins (*Bacillus subtilis*); CAB13278 [gi|2633776|emb|CAB13278.1] similar to hypothetical proteins (*Bacillus subtilis*); CAB14172 [gi|2634674|emb|CAB14172.1] menaquinol:cytochrome c oxidoreductase (iron (sulfur subunit) (*Bacillus subtilis*); CAB15089 [gi|2635595|emb|CAB15089.1] yubF (*Bacillus subtilis*); and CAB15852 [gi|2636361|emb|CAB15852.1] alternate gene name: ipa (29d—similar to hypothetical proteins (*Bacillus subtilis*)), the sequences of which are all incorporated by reference herein. Thus, in some embodiments, the signal peptide encoded by the polynucleotide in the recombinant nucleic acid molecule and/or the expression cassettes is a Tat signal peptide derived from *B. subtilis*. Information on Tat signal peptides from *Pseudomonas aeruginosa* is provided in Ochsner et al., *PNAS*, 99: 8312-8317 (2002). Also, Tat signal peptides from a wide variety of other bacteria are described in Dilks et al., *J. of Bacteriology*, 185: 1478-1483 (2003) and Berks et al., *Molecular Microbiology*, 35:260-274 (2000), both of which are incorporated by reference herein in their entirety.

[0160] Additional Tat signal peptide may be identified and distinguished from Sec-type signal peptides by their “twin-arginine” consensus motif. As noted above, signal peptides related to proteins secreted by the Tat pathway have a tripartite organization similar to Sec signal peptides, but are characterized by having an RR-motif (R—R—X—#, where # is a hydrophobic residue) located at the N-domain/H-domain boundary. Tat signal peptides are also generally longer and less hydrophobic than the Sec-type signal peptides. See, e.g., Berks et al., *Adv. Microb. Physiol.*, 47:187-254 (2003) and Berks et al., *Mol. Microbiol.* 35:260-74 (2000).

[0161] In addition, techniques analogous to those described above for the identifying new proteins secreted by the SecA2 pathway and their corresponding SecA2 signal peptides can also be used to identify new proteins secreted via the Tat pathway and their signal peptides. The reference Jongbloed et al., *J. Biological Chem.*, 277:44068-44078 (2002) provides examples of techniques which can be used to identify a protein expressed by a type of bacteria as a protein secreted via the twin-arginine translocation pathway.

[0162] IV. Polypeptides

[0163] The recombinant nucleic acid molecules described herein, as well as the expression cassettes or expression vectors described herein, can be used to encode any desired polypeptide. In particular, the recombinant nucleic acid molecules, expression cassettes, and expression vectors are useful for expressing heterologous polypeptides in a bacterium.

[0164] In some embodiments (depending on the recombinant nucleic acid molecule, expression cassette or expression vector used), the polypeptide encoded by a polynucle-

otide of the recombinant nucleic acid molecule, expression cassette, and/or expression vector is encoded as part of a fusion protein with a signal peptide. In other embodiments, the encoded polypeptide is encoded as a discrete polypeptide by the recombinant nucleic acid molecule, expression cassette, or expression vector. In still other embodiments, the polypeptide encoded by a polynucleotide of the recombinant nucleic acid molecule, expression cassette, or expression vector is encoded as part of a fusion protein that does not include a signal peptide. In still other embodiments, the polypeptide encoded by a polynucleotide of the recombinant nucleic acid molecule, expression cassette, or expression vector of the invention is encoded as part of a fusion protein (also referred to herein as a protein chimera) in which the polypeptide is embedded within another polypeptide sequence.

[0165] Thus, it is understood that each of the polypeptides listed herein (below and elsewhere) which are encoded by polynucleotides of the recombinant nucleic acid molecules, expression cassettes, or expression vectors of the invention may be expressed as either fusion proteins (fused to signal peptides and/or to or in other polypeptides) or as discrete polypeptides by the recombinant nucleic acid molecule, expression cassette, or expression vector, depending on the particular recombinant nucleic acid molecule, expression cassette or expression vector used. For instance, in some embodiments, a recombinant nucleic acid molecule comprising a polynucleotide encoding the antigen CEA will encode CEA as a fusion protein with a signal peptide.

[0166] In some embodiments, the polypeptide is part of a fusion protein encoded by the recombinant nucleic acid molecule, expression cassette, or expression vector and is heterologous to the signal peptide of the fusion protein. In some embodiments, the polypeptide is positioned within another polypeptide sequence (e.g., a secreted protein or an autolysin, or fragments or variants thereof) to which it is heterologous.

[0167] In some embodiments, the polypeptide is bacterial (either Listerial or non-Listerial). In some embodiments, the polypeptide is not bacterial. In some embodiments, the polypeptide encoded by the polynucleotide is a mammalian polypeptide. For instance, the polypeptide may correspond to a polypeptide sequence found in humans (i.e., a human polypeptide). In some embodiments, the polypeptide is Listerial. In some embodiments, the polypeptide is non-Listerial. In some embodiments, the polypeptide is not native (i.e., is foreign) to the bacterium in which the recombinant nucleic acid molecule, expression cassette, and/or expression vector is to be incorporated or is incorporated.

[0168] In some embodiments, the polynucleotide encoding the polypeptide is codon-optimized for expression in a bacterium. In some embodiments, the polynucleotide encoding the polypeptide is fully codon-optimized for expression in a bacterium. In some embodiments, the polypeptide which is encoded by the codon-optimized polynucleotide is foreign to the bacterium (i.e., is heterologous to the bacterium).

[0169] The term “polypeptide” is used interchangeably herein with “peptide” and “protein” and no limitation with respect to the length or size of the amino acid sequence contained therein is intended. Typically, however, the polypeptide will comprise at least about 6 amino acids. In

some embodiments, the polypeptide will comprise, at least about 9, at least about 12, at least about 20, at least about 30, or at least about 50 amino acids. In some embodiments, the polypeptide comprises at least about 100 amino acids. In some embodiments, the polypeptide is one particular domain of a protein (e.g., an extracellular domain, an intracellular domain, a catalytic domain, or a binding domain). In some embodiments, the polypeptide comprises an entire (i.e., full-length) protein.

[0170] In some embodiments, the polypeptide that is encoded by a polynucleotide of a recombinant nucleic acid molecule, expression cassette, and/or expression vector comprises an antigen or a protein that provides a palliative treatment for a disease. In some embodiments, the polypeptide that is encoded by a polynucleotide of a recombinant nucleic acid molecule, expression cassette, and/or expression vector is an antigen or a protein that provides a palliative treatment for a disease. In some embodiments, the polypeptide that is encoded is a therapeutic protein (or comprises a therapeutic protein).

[0171] In some embodiments, the polypeptide that is encoded by a polynucleotide of a recombinant nucleic acid molecule, expression cassette, and/or vector comprises an antigen (e.g., any of the antigens described herein). In some embodiments, the polypeptide that is encoded by a polynucleotide of a recombinant nucleic acid molecule, expression cassette, and/or vector is an antigen. In some embodiments, the antigen is a bacterial antigen. In some embodiments, the antigen is a non-Listerial bacterial antigen. In some embodiments, however, the antigen is a non-Listerial antigen. In other embodiments, the antigen is a non-bacterial antigen. In some embodiments, the antigen is a mammalian antigen. In some embodiments, the antigen is a human antigen. In some embodiments, the polypeptide is (or comprises) an antigen comprising one or more immunogenic epitopes. In some embodiments, the antigen comprises one or more MHC class I epitopes. In other embodiments, the antigen comprises one or more MHC class II epitopes. In some embodiments, the epitope is a CD4+ T-cell epitope. In other embodiments, the epitope is a CD8+ T-cell epitope.

[0172] The polynucleotide encoding an antigen is not limited to any exact nucleic acid sequence (e.g., that encoding a naturally occurring, full-length antigen) but can be of any sequence that encodes a polypeptide that is sufficient to elicit the desired immune response when administered to an individual within the bacteria or compositions of the invention. The term "antigen," as used herein, is also understood to include fragments of larger antigen proteins so long as the fragments are antigenic (i.e., immunogenic). In addition, in some embodiments, the antigen encoded by a polynucleotide of the recombinant nucleic acid, expression cassette, or expression vector may be a variant of a naturally occurring antigen sequence. (Similarly for polynucleotides encoding other, non-antigen proteins, the sequences of the polynucleotides encoding a given protein may vary so long as the desired protein that is expressed provides the desired effect (e.g. a palliative effect) when administered to an individual.)

[0173] An antigen that is derived from another antigen includes an antigen that is an antigenic (i.e., immunogenic) fragment of the other antigen, an antigenic variant of the other antigen, or an antigenic variant of a fragment of the

other antigen. A variant of an antigen includes antigens that differ from the original antigen in one or more substitutions, deletions, additions, and/or insertions.

[0174] The antigenic fragment may be of any length, but is most typically at least about 6 amino acids, at least about 9 amino acids, at least about 12 amino acids, at least about 20 amino acids, at least about 30 amino acids, at least about 50 amino acids, or at least about 100 amino acids. An antigenic fragment of an antigen comprises at least one epitope from the antigen. In some embodiments, the epitope is a MHC class I epitope. In other embodiments, the epitope is a MHC class II epitope. In some embodiments, the epitope is a CD4+ T-cell epitope. In other embodiments, the epitope is a CD8+ T-cell epitope.

[0175] A variety of algorithms and software packages useful for predicting antigenic regions (including epitopes) within proteins are available to those skilled in the art. For instance, algorithms that can be used to select epitopes that bind to MHC class I and class II molecules are publicly available. For instance, the publicly available "SYFPEITHI" algorithm can be used to predict MHC-binding peptides (Rammensee et al. (1999) Immunogenetics 50:213-9). For other examples of publicly available algorithms, see the following references: Parker et al. (1994) J. Immunol 152:163-75; Singh and Raghava (2001) Bioinformatics 17:1236-1237; Singh and Raghava (2003) Bioinformatics 19:1009-1014; Mallios (2001) Bioinformatics 17:942-8; Nielsen et al. (2004) Bioinformatics 20:1388-97; Donnes et al. (2002) BMC Bioinformatics 3:25; Bhasin, et al. (2004) Vaccine 22:3195-204; Guan et al. (2003) Nucleic Acids Res 31:3621-4; Reche et al. (2002) Hum. Immunol. 63:701-9; Schirle et al. (2001) J. Immunol Methods 257:1-16; Nussbaum et al. (2001) Immunogenetics (2001) 53:87-94; Lu et al. (2000) Cancer Res. 60:5223-7. See also, e.g., Vector NTI® Suite (Informax, Inc., Bethesda, Md.), GCG Wisconsin Package (Accelrys, Inc., San Diego, Calif.), Welling, et al. (1985) FEBS Lett. 188:215-218, Parker, et al. (1986) Biochemistry 25:5425-5432, Van Regenmortel and Pellequer (1994) Pept. Res. 7:224-228, Hopp and Woods (1981) PNAS 78:3824-3828, and Hopp (1993) Pept. Res. 6:183-190. Some of the algorithms or software packages discussed in the references listed above in this paragraph are directed to the prediction of MHC class I and/or class II binding peptides or epitopes, others to identification of proteasomal cleavage sites, and still others to prediction of antigenicity based on hydrophilicity.

[0176] Once a candidate antigenic fragment believed to contain at least one epitope of the desired nature has been identified, the polynucleotide sequence encoding that sequence can be incorporated into an expression cassette and introduced into a *Listeria* vaccine vector or other bacterial vaccine vector. The immunogenicity of the antigenic fragment can then be confirmed by assessing the immune response generated by the *Listeria* or other bacteria expressing the fragments. Standard immunological assays such as ELISPOT assays, Intracellular Cytokine Staining (ICS) assay, cytotoxic T-cell activity assays, or the like, can be used to verify that the fragment of the antigen chosen maintains the desired immunogenicity. Examples of these types of assays are provided in the Examples below (see, e.g., Example 21). In addition, the anti-tumor efficacy of the *Listeria* and/or bacterial vaccines can also be assessed using the methods described below in the Examples (e.g., implan-

tation of CT26 murine colon cells expressing the antigen fragment in mice, followed by vaccination of the mice with the candidate vaccine and observation of effect on tumor size, metastasis, survival, etc. relative to controls and/or the full-length antigen).

[0177] In addition, large databases containing epitope and/or MHC ligand information using for identifying antigenic fragments are publicly available. See, e.g., Brusic et al. (1998) *Nucleic Acids Res.* 26:368-371; Schonbach et al. (2002) *Nucleic Acids Research* 30:226-9; and Bhasin et al. (2003) *Bioinformatics* 19:665-666; and Rammensee et al. (1999) *Immunogenetics* 50:213-9.

[0178] The amino acid sequence of an antigenic variant has at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or at least about 98% identity to the original antigen.

[0179] In some embodiments, the antigenic variant is a conservative variant that has at least about 80% identity to the original antigen and the substitutions between the sequence of the antigenic variant and the original antigen are conservative amino acid substitutions. The following substitutions are considered conservative amino acid substitutions: valine, isoleucine, or leucine are substituted for alanine; lysine, glutamine, or asparagine are substituted for arginine; glutamine, histidine, lysine, or arginine are substituted for asparagine; glutamic acid is substituted for aspartic acid; serine is substituted for cysteine; asparagine is substituted for glutamine; aspartic acid is substituted for glutamic acid; proline or alanine is substituted for glycine; asparagine, glutamine, lysine or arginine is substituted for histidine; leucine, valine, methionine, alanine, phenylalanine, or norleucine is substituted for isoleucine; norleucine, isoleucine, valine, methionine, alanine, or phenylalanine is substituted for leucine; arginine, glutamine, or asparagine is substituted for lysine; leucine, phenylalanine, or isoleucine is substituted for methionine; leucine, valine, isoleucine, alanine, or tyrosine is substituted for phenylalanine; alanine is substituted for proline; threonine is substituted for serine; serine is substituted for threonine; tyrosine or phenylalanine is substituted for tryptophan; tryptophan, phenylalanine, threonine, or serine is substituted for tyrosine; tryptophan, phenylalanine, threonine, or serine is substituted for tyrosine; isoleucine, leucine, methionine, phenylalanine, alanine, or norleucine is substituted for valine. In some embodiments, the antigenic variant is a conservative variant that has at least about 90% identity to the original antigen.

[0180] In some embodiments, an antigen derived from another antigen is substantially equivalent to the other antigen. An antigen derived from another antigen is substantially equivalent to the original antigen from which it is derived if the antigen if the derived antigen has at least about 70% identity in amino acid sequence to the original antigen and maintains at least about 70% of the immunogenicity of the original antigen. In some embodiments, the substantially equivalent antigen has at least about 80%, at least about 90%, at least about 95%, or at least about 98% identity in amino acid sequence to the original antigen. In some embodiments, the substantially equivalent antigen comprises only conservative substitutions relative to the original antigen. In some embodiments, the substantially equivalent antigen maintains at least about 80%, at least about 90%, or at least about 95% of the immunogenicity of the original

antigen. To determine the immunogenicity of a particular derived antigen and compare to that of the original antigen to determine whether the derived antigen is substantially equivalent to the original antigen, one can test both the derived and original antigen in any of a number of immunogenicity assays known to those skilled in the art. For instance, *Listeria* expressing either the original antigen or the derived antigen can be prepared as described herein. The ability of those *Listeria* expressing the different antigens to produce an immune response can be measured by vaccinating mice with the *Listeria* and then assessing the immunogenic response using the standard techniques of ELISPOT assays, Intracellular Cytokine Staining (ICS) assay, cytotoxic T-cell activity assays, or the like. Examples of these types of assays are provided in the examples below (see, e.g., Example 21).

[0181] In some embodiments, the polypeptide encoded by a polynucleotide of the recombinant nucleic acid molecule, expression cassette, and/or vector comprises an antigen. In some embodiments, the antigen is selected from the group consisting of a tumor-associated antigen, a polypeptide derived from a tumor-associated antigen, an infectious disease antigen, and a polypeptide derived from an infectious disease antigen.

[0182] In some embodiments, the polypeptide encoded by a polynucleotide of the recombinant nucleic acid molecule, expression cassette, and/or vector comprises a tumor-associated antigen or comprises an antigen derived from a tumor-associated antigen. In some embodiments, the polypeptide comprises a tumor-associated antigen. In some embodiments, the encoded polypeptide comprises more than one antigen that is a tumor-associated antigen or an antigen derived from a tumor-associated antigen. For instance, in some embodiments, the encoded polypeptide comprises both mesothelin (or an antigenic fragment or antigenic variant thereof) and K-Ras, 12-K-Ras, or PSCA (or an antigenic fragment or antigenic variant of K-Ras, 12-K-Ras, or PSCA).

[0183] In some embodiments, the antigen encoded by a polynucleotide of the recombinant nucleic acid molecule, expression cassette, and/or expression vector is a tumor-associated antigen or is an antigen that is derived from a tumor-associated antigen. In some embodiments, the antigen is a tumor-associated antigen.

[0184] In some embodiments, a polynucleotide in a recombinant nucleic acid molecule, expression cassette, and/or expression vector encodes an antigen (or encodes a polypeptide comprising an antigen) that is not identical to a tumor-associated antigen, but rather is an antigen derived from a tumor-associated antigen. For instance, in some embodiments, the antigen encoded by a polynucleotide of a recombinant nucleic acid molecule, expression cassette, and/or expression vector may comprise a fragment of a tumor-associated antigen, a variant of a tumor-associated antigen, or a variant of a fragment of a tumor-associated antigen. In some cases, an antigen, such as a tumor antigen, is capable of inducing a more significant immune response in a vaccine when the amino acid sequence differs slightly from that endogenous to a host. In other cases, the derived antigen induces a less significant immune response than the original antigen, but is, for instance, more convenient for heterologous expression in a *Listeria* vaccine vector due to

a smaller size. In some embodiments, the amino acid sequence of a variant of a tumor-associated antigen, or a variant of a fragment of a tumor-associated antigen, differs from that of the tumor-associated antigen, or its corresponding fragment, by one or more amino acids. The antigen derived from a tumor-associated antigen will comprise at least one epitope sequence capable of inducing the desired immune response upon expression of the polynucleotide encoding the antigen within a host.

[0185] Accordingly, in some embodiments, a polynucleotide in the recombinant nucleic acid molecule, expression cassette, or vector encodes a polypeptide that comprises an antigen derived from a tumor-associated antigen, wherein the antigen comprises at least one antigenic fragment of a tumor-associated antigen. In some embodiments, a polynucleotide in the recombinant nucleic acid molecule, expression cassette, or vector encodes an antigen that is derived from a tumor-associated antigen, wherein the antigen comprises at least one antigenic fragment of a tumor-associated antigen. The antigenic fragment comprises at least one epitope of the tumor-associated antigen. In some embodiments, the antigen that is derived from another antigen is an antigenic (i.e., immunogenic) fragment or an antigenic variant of the other antigen. In some embodiments, the antigen is an antigenic fragment of the other antigen. In some embodiments, the antigen is an antigenic variant of the other antigen.

[0186] A large number of tumor-associated antigens that are recognized by T cells have been identified (Renkvist et al., *Cancer Immunol Immunother* 50:3-15 (2001)). These tumor-associated antigens may be differentiation antigens (e.g., PSMA, Tyrosinase, gp100), tissue-specific antigens (e.g. PAP, PSA), developmental antigens, tumor-associated viral antigens (e.g. HPV 16 E7), cancer-testis antigens (e.g. MAGE, BAGE, NY-ESO-1), embryonic antigens (e.g. CEA, alpha-fetoprotein), oncoprotein antigens (e.g. Ras, p53), over-expressed protein antigens (e.g. ErbB2 (Her2/Neu), MUC1), or mutated protein antigens. The tumor-associated antigens that may be encoded by the heterologous nucleic acid sequence include, but are not limited to, 707-AP, Annexin II, AFP, ART-4, BAGE, β -catenin/m, BCL-2, bcr-abl, bcr-abl p190, bcr-abl p210, BRCA-1, BRCA-2, CAMEL, CAP-1, CASP-8, CDC27/m, CDK-4/m, CEA (Huang et al., *Exper Rev Vaccines* (2002)1:49-63), CT9, CT10, Cyp-B, Dek-cain, DAM-6 (MAGE-B2), DAM-10 (MAGE-B1), EphA2 (Zantek et al., *Cell Growth Differ.* (1999) 10:629-38; Carles-Kinch et al., *Cancer Res.* (2002) 62:2840-7), ELF2M, EphA2 (Zantek et al., *Cell Growth Differ.* (1999) 10:629-38; Carles-Kinch et al., *Cancer Res.* (2002) 62:2840-7), ETV6-AML1, G250, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7B, GAGE-8, GnT-V, gp100, HAGE, HER2/neu, HLA-A*0201-R1701, HPV-E7, H-Ras, HSP70-2M, HST-2, hTERT, hTERT, iCE, inhibitors of apoptosis (e.g. survivin), KIAA0205, K-Ras, 12-K-Ras (K-Ras with codon 12 mutation), LAGE, LAGE-1, LDLR/FUT, MAGE-1, MAGE-2, MAGE-3, MAGE-6, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, MAGE-B5, MAGE-B6, MAGE-C2, MAGE-C3, MAGE-D, MART-1, MART-1/Melan-A, MC1R, MDM-2, mesothelin, Myosin/m, MUC1, MUC2, MUM-1, MUM-2, MUM-3, neo-polyA polymerase, NA88-A, N-Ras, NY-ESO-1, NY-ESO-1a (CAG-3), PAGE-4, PAP, Proteinase 3 (PR3) (Molldrem et al., *Blood* (1996) 88:2450-7; Molldrem et al., *Blood* (1997)

90:2529-34), P15, p190, Pml/RAR α , PRAME, PSA, PSM, PSMA, RAGE, RAS, RCAS1, RU1, RU2, SAGE, SART-1, SART-2, SART-3, SP17, SPAS-1, TEL/AML1, TPI/m, Tyrosinase, TARP, TRP-1 (gp75), TRP-2, TRP-2/INT2, WT-1, and alternatively translated NY-ESO-ORF2 and CAMEL proteins, derived from the NY-ESO-1 and LAGE-1 genes.

[0187] In some embodiments, the antigen encoded by the polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or vector may encompass any tumor-associated antigen that can elicit a tumor-specific immune response, including antigens yet to be identified. In some embodiments, the polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or vector encodes more than one tumor-associated antigen.

[0188] In some embodiments, the antigen is mesothelin (Argani et al., *Clin Cancer Res.* 7(12):3862-8 (2001)), Sp17 (Lim et al., *Blood* 97(5):1508-10 (2001)), gp100 (Kawakami et al., *Proc. Natl. Acad. Sci. USA* 91:6458 (1994)), PAGE-4 (Brinkmann et al., *Cancer Res.* 59(7):1445-8 (1999)), TARP (Wolfgang et al., *Proc. Natl. Acad. Sci. USA* 97(17):9437-42 (2000)), EphA2 (Tatsumi et al., *Cancer Res.* 63(15):4481-9 (2003)), PR3 (Muller-Berat et al., *Clin. Immunol. Immunopath.* 70(1):51-9 (1994)), prostate stem cell antigen (PSCA) (Reiter et al., *Proc. Natl. Acad. Sci.*, 95:1735-40 (1998); Kiessling et al., *Int. J. Cancer*, 102:390-7 (2002)), or SPAS-1 (U.S. patent application Publication No. 2002/0150588).

[0189] In some embodiments of the invention, the antigen encoded by the recombinant nucleic acid molecule or expression cassette is CEA. In other embodiments, the antigen is an antigenic fragment and/or antigenic variant of CEA. CEA is a 180-kDa membrane intercellular adhesion glycoprotein that is over-expressed in a significant proportion of human tumors, including 90% of colorectal, gastric, and pancreatic, 70% of non-small cell lung cancer, and 50% of breast cancer (Hammarstrom, *Semin. Cancer Biol.*, 9:67-81). A variety of immunotherapeutics such as anti-idiotypic monoclonal antibody mimicking CEA (Foon et al., *Clin. Cancer Res.*, 87:982-90 (1995), or vaccination using a recombinant vaccinia virus expressing CEA (Tsang et al., *J. Natl. Cancer Inst.*, 87:982-90 (1995)) have been investigated, unfortunately, however, with limited success. Nonetheless, investigators have identified a HLA*0201-restricted epitope, CAP-1(CEA605-613), that is recognized by human T cell lines that were generated from vaccinated patients. Vaccination of patients with DC pulsed with this epitope failed to induce clinical responses (Morse et al., *Clin. Cancer Res.*, 5:1331-8 (1999)). Recently, a CEA605-613 peptide agonist was identified with a heteroclitic aspartate to asparagine substitution at position 610 (CAP1-6D). Although this amino acid substitution did not alter MHC binding affinity of this peptide, the use of the altered peptide ligand (APL) resulted in improved generation of CEA-specific cytotoxic T lymphocytes (CTL) in vitro. CAP1-6D-specific CTL maintained their ability to recognize and lyse tumor cells expressing native CEA (Zaromba et al., *Cancer Res.*, 57: 4570-7 (1997); Salazar et al., *Int. J. Cancer*, 85:829-38 (2000)). Fong et al. demonstrated induction of CEA-specific immunity in patients with colon cancer vaccinated with Flt3-ligand expanded DC incubated with this APL. Encouragingly, 2 of 12 patients after vaccination experienced dramatic tumor regressions that correlated with the induction of peptide-MHC tetramer⁺ T cells (Fong et al., *Proc. Natl. Acad. Sci. U.S.A.*, 98:8809-14 (2001)).

[0190] In another embodiment, the antigen is proteinase-3 or is derived from proteinase-3. For instance, in one embodiment, the antigen comprises the HLA-A2.1-restricted peptide PR1 (aa 169-177; VLQELNVTV (SEQ ID NO:63)). Information on proteinase-3 and/or the PR1 epitope is available in the following references: U.S. Pat. No. 5,180,819, Mollidrem, et al., *Blood*, 90:2529-2534 (1997); Mollidrem et al., *Cancer Research*, 59:2675-2681 (1999); Mollidrem, et al., *Nature Medicine*, 6:1018-1023 (2000); and Mollidrem et al., *Oncogene*, 21: 8668-8673 (2002).

[0191] In some embodiments, the polypeptide encoded by a polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or vector comprises an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or comprises an antigen derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA.

[0192] In some embodiments, the polypeptide encoded by a polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or vector comprises an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. In some embodiments, the polypeptide comprises K-Ras. In some embodiments, the polypeptide comprises H-Ras. In some embodiments, the polypeptide comprises N-Ras. In some embodiments, the polypeptide comprises K-Ras. In some embodiments, the polypeptide comprises mesothelin (e.g., human mesothelin). In some embodiments, the polypeptide comprises PSCA. In some embodiments, the polypeptide comprises NY-ESO-1. In some embodiments, the polypeptide comprises WT-1. In some embodiments, the polypeptide comprises survivin. In some embodiments, the polypeptide comprises gp100. In some embodiments, the polypeptide comprises PAP. In some embodiments, the polypeptide comprises proteinase 3. In some embodiments, the polypeptide comprises SPAS-1. In some embodiments, the polypeptide comprises SP-17. In some embodiments, the polypeptide comprises PAGE-4. In some embodiments, the polypeptide comprises TARP. In some embodiments, the polypeptide comprises CEA.

[0193] In some embodiments, the antigen encoded by a polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or vector is an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. In some embodiments, the antigen is K-Ras. In some embodiments, the antigen is H-Ras. In some embodiments, the antigen is N-Ras. In some embodiments, the antigen is K-Ras. In some embodiments, the antigen is mesothelin. In some embodiments, the antigen is PSCA. In some embodiments, the antigen is NY-ESO-1. In some embodiments, the antigen is WT-1. In some embodiments, the antigen is survivin. In some embodiments, the antigen is gp100. In some embodiments, the antigen is PAP. In some embodiments, the antigen is proteinase 3. In some embodiments, the antigen is SPAS-1. In some embodiments, the antigen is SP-17. In some embodiments, the antigen is PAGE-4. In

some embodiments, the antigen is TARP. In some embodiments, the antigen is CEA. In some embodiments, the antigen is human mesothelin.

[0194] In some embodiments, the antigen is mesothelin, SPAS-1, proteinase-3, EphA2, SP-17, gp100, PAGE-4, TARP, or CEA, or an antigen derived from one of those proteins. In some embodiments the antigen is mesothelin or is derived from mesothelin. In other embodiments, the antigen is EphA2 or is an antigen derived from EphA2. In some embodiments, the antigen encoded by a polynucleotide in a recombinant nucleic acid molecule, expression cassette, or expression vector described herein is not EphA2 (or an antigen derived from EphA2). In some embodiments, the antigen is a tumor-associated antigen other than EphA2. In some embodiments, the antigen is derived from a tumor-associated antigen other than EphA2. In some embodiments, the polypeptide encoded by a polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or expression vector comprises an antigen other than EphA2. In some embodiments, the polypeptide encoded by a polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or expression vector comprises an antigen other than EphA2 or an antigen derived from EphA2.

[0195] In some embodiments, a polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or expression vector encodes a polypeptide comprising an antigen derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. In some embodiments, the polypeptide comprises an antigen derived from K-Ras. In some embodiments, the polypeptide comprises an antigen derived from H-Ras. In some embodiments, the polypeptide comprises an antigen derived from N-Ras. In some embodiments, the polypeptide comprises an antigen derived from 12-K-Ras. In some embodiments, the polypeptide comprises an antigen derived from mesothelin. In some embodiments, the polypeptide comprises an antigen derived from PSCA. In some embodiments, the polypeptide comprises an antigen derived from NY-ESO-1. In some embodiments, the polypeptide comprises an antigen derived from WT-1. In some embodiments, the polypeptide comprises an antigen derived from survivin. In some embodiments, the polypeptide comprises an antigen derived from gp100. In some embodiments, the polypeptide comprises an antigen derived from PAP. In some embodiments, the polypeptide comprises an antigen derived from proteinase 3. In some embodiments, the polypeptide comprises an antigen derived from SPAS-1. In some embodiments, the polypeptide comprises an antigen derived from SP-17. In some embodiments, the polypeptide comprises an antigen derived from PAGE-4. In some embodiments, the polypeptide comprises an antigen derived from TARP. In some embodiments, the polypeptide comprises an antigen derived from CEA.

[0196] In some embodiments, a polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or expression vector encodes an antigen derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. In some embodiments, the antigen is derived from K-Ras. In some embodiments, the antigen is derived from H-Ras. In some embodiments, the antigen is derived from N-Ras. In some embodiments, the antigen is derived from 12-K-Ras. In some embodiments,

the antigen is an antigen derived from mesothelin. In some embodiments, the antigen is an antigen derived from PSCA. In some embodiments, the antigen is an antigen derived from NY-ESO-1. In some embodiments, the antigen is an antigen derived from WT-1. In some embodiments, the antigen is an antigen derived from survivin. In some embodiments, the antigen is an antigen that is derived from gp100. In some embodiments, the antigen is an antigen that is derived from PAP. In some embodiments, the antigen is an antigen that is derived from proteinase 3. In some embodiments, the antigen is an antigen derived from SPAS-1. In some embodiments, the antigen is an antigen derived from SP-17. In some embodiments, the antigen is an antigen derived from PAGE-4. In some embodiments, the antigen is an antigen derived from TARP. In some embodiments, the antigen is an antigen derived from CEA.

[0197] In some embodiments, the antigen is mesothelin, or an antigenic fragment or antigenic variant thereof. Thus, in some embodiments, the polypeptide encoded by a polynucleotide in the recombinant nucleic acid molecule, expression cassette and/or vector comprises mesothelin, or an antigenic fragment or antigenic variant thereof. In some embodiments, the polypeptide encoded by the polynucleotide is mesothelin, or an antigenic fragment or antigenic variant thereof.

[0198] In some embodiments, the antigen is mesothelin (e.g., human mesothelin) in which the mesothelin signal peptide and/or GPI (glycosylphosphatidylinositol) anchor has been deleted. Accordingly, in some embodiments, the polypeptide encoded by the polynucleotide comprises mesothelin in which the mesothelin signal peptide and/or GPI anchor has been deleted. In some embodiments, the polypeptide encoded by the polynucleotide is mesothelin in which the mesothelin signal peptide and/or GPI anchor has been deleted. In some embodiments, the polypeptide encoded by the polynucleotide is human mesothelin in which the mesothelin signal peptide and/or GPI anchor has been deleted. In some embodiments, the polypeptide encoded by the polynucleotide is human mesothelin in which both the mesothelin signal peptide and GPI anchor have been deleted.

[0199] In some embodiments, the antigen is NY-ESO-1, or an antigenic fragment or antigenic variant thereof. Thus, in some embodiments, the polypeptide encoded by a polynucleotide in the recombinant nucleic acid molecule, expression cassette, or vector comprises an antigen which is NY-ESO-1, or an antigenic fragment or antigenic variant thereof. In some embodiments, the polypeptide is an antigen which is NY-ESO-1, or an antigenic fragment or antigenic variant thereof.

[0200] In some embodiments, a polypeptide encoded by polynucleotide in a recombinant nucleic acid molecule, expression cassette, or vector comprises at least one antigenic fragment of a tumor-associated antigen, e.g., human prostate stem cell antigen (PSCA; GenBank Acc. No. AF043498), human testes antigen (NY-ESO-1; GenBank Acc. No. NM_001327), human carcinoembryonic antigen (CEA; GenBank Acc. No. M29540), human Mesothelin (GenBank Acc. No. U40434), human survivin (GenBank Acc. No. U75285), human Proteinase 3 (GenBank No. X55668), human K-Ras (GenBank Acc. Nos. M54969 & P01116), human H-Ras (GenBank Acc. No. P01112), human

N-Ras (GenBank Acc. No. P01111), and human 12-K-Ras (K-Ras comprising a Gly12Asp mutation) (see, e.g., GenBank Acc. No. K00654). In some embodiments, a polypeptide encoded by polynucleotide in a recombinant nucleic acid molecule, expression cassette, or expression vector comprises an antigenic fragment of a tumor-associated antigen with at least one conservatively substituted amino acid. In some embodiments, a polypeptide encoded by polynucleotide in a recombinant nucleic acid molecule, expression cassette, or expression vector comprises an antigenic fragment with at least one deleted amino acid residue. In some embodiments, a polypeptide encoded by polynucleotide in a recombinant nucleic acid molecule, expression cassette, or expression vector comprises combinations of antigenic sequences derived from more than one type of tumor-associated antigen, e.g., a combination of antigenic fragments derived from both mesothelin and Ras.

[0201] Exemplary regions of tumor antigens predicted to be antigenic include the following: amino acids 25-35; 70-80; and 90-118 of the PSCA amino acid sequence in GenBank Acc. No. AF043498; amino acids 40-55, 75-85, 100-115, and 128-146 of the NY-ESO-1 of GenBank Acc. No. NM_001327; amino acids 70-75, 150-155, 205-225, 330-340, and 510-520 of the CEA amino acid sequence of GenBank Acc. No. M29540; amino acids 90-110, 140-150, 205-225, 280-310, 390-410, 420-425, and 550-575; of the mesothelin polypeptide sequence of GenBank Acc. No. U40434; amino acids 12-20, 30-40, 45-55, 65-82, 90-95, 102-115, and 115-130 of the surviving polypeptide sequence of GenBank Acc. No. U75285; amino acids 10-20, 30-35, 65-75, 110-120, and 160-170, of the amino acid sequence of proteinase-3 found in GenBank Acc. No. X55668; amino acids 10-20, 30-50, 55-75, 85-110, 115-135, 145-155, and 160-185 of GenBank Acc. Nos. P01117 or M54968 (human K-Ras); amino acids 10-20, 25-30, 35-45, 50-70, 90-110, 115-135, and 145-175 of GenBank Acc. No. P01112 (human H-Ras); amino acids 10-20, 25-45, 50-75, 85-110, 115-135, 140-155, and 160-180 of GenBank Acc. No. P01111 (human N-Ras); and the first 25-amino acids of 12-K-Ras (sequence disclosed in GenBank Acc. No. K00654). These antigenic regions were predicted by Hopp-Woods and Welling antigenicity plots.

[0202] In some embodiments, the polypeptides encoded by the polynucleotides of the invention either as discrete polypeptides, as fusion proteins with the chosen signal peptide, or as a protein chimera in which the polypeptide has been inserted in another polypeptide, are polypeptides comprising one or more of the following peptides of human mesothelin: SLLFLLFSL (amino acids 20-28; (SEQ ID NO:64)); VLPLTVAEV (amino acids 530-538; (SEQ ID NO:65)); ELAVALAQK (amino acids 83-92; (SEQ ID NO:66)); ALQGGGPPY (amino acids 225-234; (SEQ ID NO:67)); FYPGYLCSL (amino acids 435-444; (SEQ ID NO:68)); and LYPKARLAF (amino acids 475-484; (SEQ ID NO:69)). For instance, in some embodiments, the antigen encoded by a polynucleotide of the invention is an (antigenic) fragment of human mesothelin comprising one or more of these peptides. Additional information regarding these mesothelin peptide sequences and their correlation with medically relevant immune responses can be found in the PCT Publication WO 2004/006837.

[0203] Alternatively, polynucleotides in the recombinant nucleic acid molecule, expression cassette, or expression

vector can encode an autoimmune disease-specific antigen (or a polypeptide comprising an autoimmune disease-specific antigen). In a T cell mediated autoimmune disease, a T cell response to self antigens results in the autoimmune disease. The type of antigen for use in treating an autoimmune disease with the vaccines of the present invention might target the specific T cells responsible for the autoimmune response. For example, the antigen may be part of a T cell receptor, the idiotype, specific to those T cells causing an autoimmune response, wherein the antigen incorporated into a vaccine of the invention would elicit an immune response specific to those T cells causing the autoimmune response. Eliminating those T cells would be the therapeutic mechanism to alleviating the autoimmune disease. Another possibility would be to incorporate into the recombinant nucleic acid molecule a polynucleotide encoding an antigen that will result in an immune response targeting the antibodies that are generated to self antigens in an autoimmune disease or targeting the specific B cell clones that secrete the antibodies. For example, a polynucleotide encoding an idiotype antigen may be incorporated into the recombinant nucleic acid molecule that will result in an anti-idiotypic immune response to such B cells and/or the antibodies reacting with self antigens in an autoimmune disease. Autoimmune diseases treatable with vaccines comprising bacteria comprising the expression cassettes and recombinant nucleic acid molecules of the present invention include, but are not limited to, rheumatoid arthritis, multiple sclerosis, Crohn's disease, lupus, myasthenia gravis, vitiligo, scleroderma, psoriasis, pemphigus vulgaris, fibromyalgia, colitis and diabetes. A similar approach may be taken for treating allergic responses, where the antigens incorporated into the vaccine bacterium target either T cells, B cells or antibodies that are effective in modulating the allergic reaction. In some autoimmune diseases, such as psoriasis, the disease results in hyperproliferative cell growth with expression of antigens that may be targeted as well. Such an antigen that will result in an immune response to the hyperproliferative cells is considered.

[0204] In some embodiments, the antigen is an antigen that targets unique disease associated protein structures. One example of this is the targeting of antibodies, B cells or T cells using idiotype antigens as discussed above. Another possibility is to target unique protein structures resulting from a particular disease. An example of this would be to incorporate an antigen that will generate an immune response to proteins that cause the amyloid plaques observed in diseases such as Alzheimer's disease, Creutzfeldt-Jakob disease (CJD) and Bovine Spongiform Encephalopathy (BSE). While this approach may only provide for a reduction in plaque formation, it may be possible to provide a curative vaccine in the case of diseases like CJD. This disease is caused by an infectious form of a prion protein. In some embodiments, the polynucleotides of the invention encode an antigen to the infectious form of the prion protein such that the immune response generated by the vaccine may eliminate, reduce, or control the infectious proteins that cause CJD.

[0205] In some embodiments, the polypeptide encoded by a polynucleotide of the recombinant nucleic acid molecule, expression cassette, and/or expression vector comprises an infectious disease antigen or an antigen derived from an infectious disease antigen. In some embodiments, the polypeptide comprises an infectious disease antigen. In

some other embodiments, the polypeptide comprises an antigen derived from an infectious disease antigen. In some embodiments, the polypeptide encoded by a polynucleotide of the recombinant nucleic acid molecule, expression cassette, and/or expression vector is an infectious disease antigen or is an antigen derived from an infectious disease antigen. In some embodiments, the polypeptide encoded by the recombinant nucleic acid molecule, expression cassette, and/or expression vector is an infectious disease antigen. In some embodiments, the polypeptide encoded by the recombinant nucleic acid molecule, expression cassette, and/or expression vector is derived from an infectious disease antigen.

[0206] In other embodiments of the invention, the antigen is derived from a human or animal pathogen. The pathogen is optionally a virus, bacterium, fungus, or a protozoan. For instance, the antigen may be a viral or fungal or bacterial antigen. In one embodiment, the antigen encoded by the recombinant nucleic acid molecule, expression cassette, and/or expression vector that is derived from the pathogen is a protein produced by the pathogen, or is derived from a protein produced by the pathogen. For instance, in some embodiments, the polypeptide encoded by the recombinant nucleic acid molecules, expression cassette and/or expression vector is a fragment and/or variant of a protein produced by the pathogen.

[0207] For instance, in some embodiments, the antigen is derived from Human Immunodeficiency virus (such as gp 120, gp 160, gp41, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol, env, tat, vif, rev, nef, vpr, vpu and LTR regions of HIV), Feline Immunodeficiency virus, or human or animal herpes viruses. For example, in some embodiments, the antigen is gp 120. In one embodiment, the antigen is derived from herpes simplex virus (HSV) types 1 and 2 (such as gD, gB, gH, Immediate Early protein such as ICP27), from cytomegalovirus (such as gB and gH), from metapneumovirus, from Epstein-Barr virus or from Varicella Zoster Virus (such as gpI, II or III). (See, e. g., Chec et al. (1990) *Cytomegaloviruses* (J. K. McDougall, ed., Springer Verlag, pp. 125-169; McGeoch et al. (1988) *J. Gen. Virol.* 69: 1531-1574; U.S. Pat. No. 5,171,568; Baer et al. (1984) *Nature* 310: 207-211; and Davison et al. (1986) *J. Gen. Virol.* 67: 1759-1816.)

[0208] In another embodiment, the antigen is derived from a hepatitis virus such as hepatitis B virus (for example, Hepatitis B Surface antigen), hepatitis A virus, hepatitis C virus, delta hepatitis virus, hepatitis E virus, or hepatitis G virus. See, e. g., WO 89/04669; WO 90/11089; and WO 90/14436. The hepatitis antigen can be a surface, core, or other associated antigen. The HCV genome encodes several viral proteins, including E1 and E2. See, e. g., Houghton et al., *Hepatology* 14: 381-388 (1991).

[0209] An antigen that is a viral antigen is optionally derived from a virus from any one of the families Picornaviridae (e. g., polioviruses, rhinoviruses, etc.); Caliciviridae; Togaviridae (e. g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae (e. g., rotavirus, etc.); Birnaviridae; Rhabdoviridae (e. g., rabies virus, etc.); Orthomyxoviridae (e. g., influenza virus types A, B and C, etc.); Filoviridae; Paramyxoviridae (e. g., mumps virus, measles virus, respiratory syncytial virus, parainfluenza virus, etc.); Bunyaviridae; Arenaviridae; Retroviridae (e. g.,

HTLV-I; HTLV-11; HIV-1 (also known as HTLV-111, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV111b, HIVSF2, HTVLAV, HIVLAI, HIVMN; HIV-1CM235, HIV-1; HIV-2, among others; simian immunodeficiency virus (SIV); Papillomavirus, the tick-borne encephalitis viruses; and the like. See, e.g. *Virology*, 3rd Edition (W. K. Joklik ed. 1988); *Fundamental Virology*, 3rd Edition (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds. 1996), for a description of these and other viruses. In one embodiment, the antigen is Flu-HA (Morgan et al., *J. Immunol.* 160:643 (1998)).

[0210] In some alternative embodiments, the antigen is derived from bacterial pathogens such as *Mycobacterium*, *Bacillus*, *Yersinia*, *Salmonella*, *Neisseria*, *Borrelia* (for example, OspA or OspB or derivatives thereof), *Chlamydia*, or *Bordetella* (for example, P.69, PT and FHA), or derived from parasites such as plasmodium or *Toxoplasma*. In one embodiment, the antigen is derived from *Mycobacterium tuberculosis* (e.g. ESAI-6, 85A, 85B, 85C, 72F), *Bacillus anthracis* (e.g. PA), or *Yersinia pestis* (e.g. F1, V). In addition, antigens suitable for use in the present invention can be obtained or derived from known causative agents responsible for diseases including, but not limited to, Diphtheria, Pertussis, Tetanus, Tuberculosis, Bacterial or Fungal Pneumonia, Otitis Media, Gonorrhea, Cholera, Typhoid, Meningitis, Mononucleosis, Plague, Shigellosis or Salmonellosis, Legionaire's Disease, Lyme Disease, Leprosy, Malaria, Hookworm, Onchocerciasis, Schistosomiasis, Trypanosomiasis, Leishmaniasis, Giardia, Amoebiasis, Filariasis, Borelia, and Trichinosis. Still further antigens can be obtained or derived from unconventional pathogens such as the causative agents of kuru, Creutzfeldt-Jakob disease (CJD), scrapie, transmissible mink encephalopathy, and chronic wasting diseases, or from proteinaceous infectious particles such as prions that are associated with mad cow disease.

[0211] In still other embodiments, the antigen is obtained or derived from a biological agent involved in the onset or progression of neurodegenerative diseases (such as Alzheimer's disease), metabolic diseases (such as Type I diabetes), and drug addictions (such as nicotine addiction). Alternatively, the antigen encoded by the recombinant nucleic acid molecule is used for pain management and the antigen is a pain receptor or other agent involved in the transmission of pain signals.

[0212] In some embodiments, the antigen is a human protein or is derived from a human protein. In other embodiments, the antigen is a non-human protein or is derived from a non-human protein (a fragment and/or variant thereof). In some embodiments, the antigen portion of the fusion protein encoded by the expression cassette is a protein from a non-human animal or is a protein derived from a non-human animal. For instance, even if the antigen is to be expressed in a *Listeria*-based vaccine that is to be used in humans, in some embodiments, the antigen can be murine mesothelin or derived from murine mesothelin.

[0213] V. Codon-Optimization

[0214] In some embodiments, one or more of the polynucleotides (i.e., polynucleotide sequences) within the recombinant nucleic acid molecule, expression cassette and/or expression vector are codon-optimized (relative to the native coding sequence). In some embodiments, a poly-

nucleotide in the recombinant nucleic acid molecules (and/or in the expression cassette and/or expression vector) described herein that encodes a signal peptide is codon-optimized for expression in a bacterium. In some embodiments, a polynucleotide encoding a polypeptide other than a signal peptide, such as an antigen or other therapeutic protein, is codon-optimized for expression in a bacterium. In some embodiments, both a polynucleotide encoding a signal peptide and a polynucleotide encoding another polypeptide fused to the signal peptide are codon-optimized for expression in a bacterium. In some embodiments, a polynucleotide encoding a secreted protein (or fragment thereof) used as a scaffold or a polynucleotide encoding an autolysin (or fragment or variant thereof) is codon-optimized.

[0215] A polynucleotide comprising a coding sequence is "codon-optimized" if at least one codon of the native coding sequence of the polynucleotide has been replaced with a codon that is more frequently used by the organism in which the coding sequence is to be expressed (the "target organism") than the original codon of the native coding sequence. For instance, a polynucleotide encoding a non-bacterial antigen that is to be expressed in a particular species of bacteria is codon-optimized if at least one of the codons from the native bacterial polynucleotide sequence is replaced with a codon that is preferentially expressed in that particular species of bacteria in which the non-bacterial antigen is to be expressed. As another example, a polynucleotide encoding a human cancer antigen that is to be part of an expression cassette in recombinant *Listeria monocytogenes* is codon-optimized if at least one codon in the polynucleotide sequence is replaced with a codon that is more frequently used by *Listeria monocytogenes* for that amino acid than the codon in the original human sequence would be. Likewise, a polynucleotide encoding a signal peptide native to *Listeria monocytogenes* (such as the LLO signal peptide from *L. monocytogenes*) that is to be part of an expression cassette to encode a fusion protein comprising a human cancer antigen in recombinant *Listeria monocytogenes* is codon-optimized if at least one codon in the polynucleotide sequence encoding the signal peptide is replaced with a codon that is more frequently used by *Listeria monocytogenes* for that amino acid than the codon in the original (native) sequence is. In some embodiments, at least one codon that is replaced in the codon-optimized sequence is replaced with the codon most frequently used by the target organism to code for the same amino acid.

[0216] In some embodiments, at least two codons of the native coding sequence of the polynucleotide have been replaced with a codon that is more frequently used by the organism in which the coding sequence is to be expressed than the original codon of the native coding sequence. In some embodiments, at least about five codons, at least about 10 codons, or at least about 20 codons of the native coding sequence of the polynucleotide have been replaced with a codon that is more frequently used by the organism in which the coding sequence is to be expressed than the original codon of the native coding sequence.

[0217] In some embodiments, at least about 10% of the codons in the codon-optimized polynucleotide have been replaced with codons more frequently (or most frequently) used by the target organism (than the original codons of the native sequence). In other embodiments, at least about 25% of the codons in the codon-optimized polynucleotide have

been replaced with codons more frequently used (or most frequently) used by the target organism. In other embodiments, at least about 50% of the codons in the codon-optimized polynucleotide have been replaced with codons more frequently used (or most frequently) used by the target organism. In still other embodiments, at least about 75% of the codons in the codon-optimized polynucleotide have been replaced with codons more frequently used (or most frequently used) by the target organism.

[0218] The codon preferences of different organisms have been widely studied by those skilled in the art. For instance, see Sharp et al., *Nucleic Acids Res.*, 15:1281-95 (1987) and Uchijima et al., *The Journal of Immunology*, 161:5594-9 (1998). As a result, codon usage tables are publicly available

for a wide variety of organisms. For instance, codon usage tables can be found on the internet at www.kazusa.or.jp/codon/ for a wide variety of organisms as well as on other publicly available sites. (See, e.g., Nakamura et al. (2000) *Nucleic Acids Research* 28:292.) An exemplary codon usage table from www.kazusa.or.jp/codon/, the codon usage table for *Listeria monocytogenes* ([http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Listeria+monocytogenes+\[gbt\]](http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Listeria+monocytogenes+[gbt])), is reproduced for convenience below in Table 2A. Exemplary codon usage tables for *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Mycobacterium bovis* BCG, and *Shigella flexneri* are also provided in Tables 2B, 2C, 2D, 2E, and 2F, respectively, below.

TABLE 2A

Codon Usage Table for <i>Listeria Monocytogenes</i> (from www.kazusa.or.jp/codon/). <i>Listeria monocytogenes</i> : 3262 CDS's (1029006 codons) fields: [triplet] [frequency: per thousand] ([number])			
UUU 29.4(30274)	UCU 13.2(13586)	UAU 22.9(23604)	UGU 3.8(3960)
UUC 14.1(14486)	UCC 6.5(6714)	UAC 10.7(11055)	UGC 1.9(1972)
UUA 36.8(37821)	UCA 10.4(10751)	UAA 2.2(2307)	UGA 0.6(583)
UUG 12.3(12704)	UCG 6.1(6278)	UAG 0.4(372)	UGG 9.3(9580)
CUU 21.0(21567)	CCU 8.4(8622)	CAU 12.0(12332)	CGU 12.6(12930)
CUC 5.4(5598)	CCC 1.7(1780)	CAC 5.2(5336)	CGC 7.0(7215)
CUA 12.9(13279)	CCA 18.5(18996)	CAA 29.9(30719)	CGA 5.6(5732)
CUG 5.0(5120)	CCG 7.0(7219)	CAG 5.1(5234)	CGG 2.8(2884)
AUU 49.3(50692)	ACU 17.1(17614)	AAU 33.0(33908)	AGU 14.1(14534)
AUC 18.4(18894)	ACC 6.9(7089)	AAC 15.3(15790)	AGC 8.8(9031)
AUA 9.4(9642)	ACA 26.5(27318)	AAA 61.6(63379)	AGA 6.9(7111)
AUG 25.9(26651)	ACG 12.9(13285)	AAG 10.4(10734)	AGG 1.2(1254)
GUU 26.4(27202)	GCU 24.3(24978)	GAU 39.8(40953)	GGU 24.2(24871)
GUC 8.7(8990)	GCC 8.4(8612)	GAC 14.3(14751)	GGC 14.2(14581)
GUA 21.6(22247)	GCA 28.6(29401)	GAA 60.4(62167)	GGA 19.1(19612)
GUG 13.1(13518)	GCG 16.6(17077)	GAG 13.1(13507)	GGG 8.7(9003)

[0219]

TABLE 2B

Codon Usage Table for <i>Bacillus anthracis</i> (from www.kazusa.or.jp/codon/). <i>Bacillus anthracis</i> [gbt]: 312 CDS's (90023 codons) fields: [triplet] [frequency: per thousand] ([number])			
UUU 32.4(2916)	UCU 17.2(1547)	UAU 31.9(2876)	UGU 5.1(455)
UUC 10.4(934)	UCC 5.0(453)	UAC 9.5(853)	UGC 1.8(164)
UUA 43.7(3931)	UCA 14.8(1330)	UAA 2.2(199)	UGA 0.5(47)
UUG 11.4(1024)	UCG 4.2(375)	UAG 0.7(66)	UGG 9.3(835)
CUU 14.4(1300)	CCU 10.7(967)	CAU 15.5(1392)	CGU 9.8(883)
CUC 3.7(335)	CCC 2.7(242)	CAC 4.2(379)	CGC 2.5(223)
CUA 12.4(1117)	CCA 17.8(1599)	CAA 32.3(2912)	CGA 6.3(569)
CUG 4.4(392)	CCG 5.9(534)	CAG 9.5(859)	CGG 2.0(179)
AUU 44.5(4009)	ACU 21.0(1890)	AAU 44.0(3959)	AGU 17.4(1565)
AUC 11.9(1072)	ACC 5.0(453)	AAC 14.1(1268)	AGC 5.2(467)
AUA 22.7(2042)	ACA 26.8(2414)	AAA 64.3(5786)	AGA 13.7(1236)
AUG 23.3(2098)	ACG 9.4(844)	AAG 22.7(2047)	AGG 4.1(368)
GUU 20.3(1824)	GCU 17.8(1598)	GAU 39.3(3536)	GGU 17.9(1611)
GUC 4.6(414)	GCC 4.1(372)	GAC 9.0(811)	GGC 5.8(524)
GUA 26.4(2374)	GCA 23.5(2117)	GAA 53.9(4855)	GGA 24.5(2203)
GUG 10.8(973)	GCG 7.9(709)	GAG 17.9(1614)	GGG 12.0(1083)

Coding GC 34.55% 1st letter GC 44.99% 2nd letter GC 33.16% 3rd letter GC 25.51%

[0220]

TABLE 2C

Codon Usage Table for *Mycobacterium tuberculosis* (from www.kazusa.or.jp/codon/).
Mycobacterium tuberculosis [gbtct]: 363 CDS's (131426 codons)
 fields: [triplet] [frequency: per thousand] ([number])

UUU 5.4(709)	UCU 2.0(265)	UAU 6.0(788)	UGU 2.5(326)
UUC 25.6(3359)	UCC 11.4(1499)	UAC 17.6(2307)	UGC 5.6(738)
UUA 1.8(231)	UCA 4.3(571)	UAA 0.4(52)	UGA 1.5(201)
UUG 14.8(1945)	UCG 19.2(2522)	UAG 0.8(103)	UGG 17.9(2352)
CUU 5.9(778)	CCU 3.9(511)	CAU 5.4(711)	CGU 8.0(1048)
CUC 17.7(2329)	CCC 18.3(2411)	CAC 14.7(1928)	CGC 26.7(3508)
CUA 4.0(521)	CCA 6.4(843)	CAA 7.8(1030)	CGA 5.8(764)
CUG 45.9(6032)	CCG 33.2(4359)	CAG 24.2(3176)	CGG 21.1(2772)
AUU 7.6(993)	ACU 4.1(545)	AAU 4.8(637)	AGU 4.0(531)
AUC 32.7(4300)	ACC 36.0(4735)	AAC 26.3(3451)	AGC 15.0(1976)
AUA 2.1(282)	ACA 4.7(616)	AAA 5.8(761)	AGA 1.5(192)
AUG 19.7(2591)	ACG 16.4(2158)	AAG 26.5(3485)	AGG 3.3(429)
GUU 8.3(1095)	GCU 11.2(1473)	GAU 15.6(2046)	GGU 18.7(2455)
GUC 32.3(4249)	GCC 51.5(6769)	GAC 44.6(5858)	GGC 48.6(6383)
GUA 4.7(622)	GCA 12.4(1625)	GAA 16.8(2211)	GGA 9.0(1183)
GUG 35.7(4687)	GCG 41.7(5482)	GAG 35.8(4702)	GGG 16.9(2215)

Coding GC 64.43% 1st letter GC 65.27% 2nd letter GC 48.28% 3rd letter GC 79.75%

[0221]

TABLE 2D

Codon Usage Table for *Salmonella typhimurium* (from www.kazusa.or.jp/codon/).
Salmonella typhimurium [gbtct]: 1322 CDS's (416065 codons)
 fields: [triplet] [frequency: per thousand] ([number])

UUU 21.7(9041)	UCU 8.5(3518)	UAU 16.5(6853)	UGU 4.6(1920)
UUC 15.1(6265)	UCC 10.6(4430)	UAC 11.6(4826)	UGC 6.1(2524)
UUA 13.6(5650)	UCA 7.9(3286)	UAA 1.8(731)	UGA 1.1(465)
UUG 12.1(5025)	UCG 9.4(3924)	UAG 0.3(121)	UGG 14.1(5851)
CUU 12.1(5038)	CCU 7.9(3290)	CAU 12.1(5047)	CGU 18.1(7542)
CUC 10.6(4396)	CCC 7.0(2921)	CAC 9.2(3818)	CGC 20.8(8659)
CUA 4.7(1958)	CCA 6.5(2712)	CAA 12.8(5315)	CGA 4.1(1695)
CUG 49.3(20508)	CCG 22.7(9463)	CAG 30.8(12803)	CGG 7.2(3004)
AUU 28.1(11700)	ACU 8.2(3401)	AAU 19.5(8107)	AGU 8.6(3569)
AUC 23.9(9941)	ACC 24.0(9980)	AAC 21.4(8920)	AGC 18.0(7485)
AUA 6.7(2771)	ACA 8.0(3316)	AAA 33.0(13740)	AGA 3.2(1348)
AUG 26.1(10842)	ACG 18.6(7743)	AAG 12.4(5151)	AGG 2.3(959)
GUU 16.4(6831)	GCU 14.4(5985)	GAU 32.9(13700)	GGU 18.1(7541)
GUC 17.7(7367)	GCC 27.5(11462)	GAC 21.5(8949)	GGC 33.0(13730)
GUA 11.9(4935)	GCA 14.8(6156)	GAA 36.1(15021)	GGA 9.1(3788)
GUG 24.3(10092)	GCG 37.0(15387)	GAG 20.9(8715)	GGG 11.6(4834)

Coding GC 52.45% 1st letter GC 58.32% 2nd letter GC 41.31% 3rd letter GC 57.71%

[0222]

TABLE 2E

Codon Usage Table for *Mycobacterium bovis*
 BCG (from www.kazusa.or.jp/codon/).
Mycobacterium bovis BCG [gbtct]: 51 CDS's (16528 codons)
 fields: [triplet] [frequency: per thousand] ([number])

UUU 4.7(77)	UCU 1.9(31)	UAU 6.6(109)	UGU 2.0(33)
UUC 27.4(453)	UCC 11.4(189)	UAC 17.0(281)	UGC 6.7(110)
UUA 1.6(26)	UCA 4.5(74)	UAA 0.9(15)	UGA 1.3(22)
UUG 14.7(243)	UCG 20.8(343)	UAG 0.8(14)	UGG 14.3(237)
CUU 5.6(92)	CCU 2.9(48)	CAU 4.9(81)	CGU 9.4(155)
CUC 14.8(244)	CCC 16.3(270)	CAC 17.2(285)	CGC 33.8(559)
CUA 5.1(85)	CCA 5.1(84)	CAA 7.3(120)	CGA 7.1(118)
CUG 51.5(852)	CCG 31.0(512)	CAG 25.5(421)	CGG 26.7(441)
AUU 6.1(100)	ACU 3.1(51)	AAU 4.8(80)	AGU 2.8(46)
AUC 39.6(654)	ACC 36.8(609)	AAC 22.3(369)	AGC 14.5(240)
AUA 2.2(37)	ACA 4.4(73)	AAA 6.2(102)	AGA 1.1(19)
AUG 20.2(334)	ACG 17.4(288)	AAG 24.5(405)	AGG 3.8(62)
GUU 7.8(129)	GCU 9.6(158)	GAU 13.4(222)	GGU 16.9(280)

TABLE 2E-continued

Codon Usage Table for *Mycobacterium bovis*
 BCG (from www.kazusa.or.jp/codon/).
Mycobacterium bovis BCG [gbtct]: 51 CDS's (16528 codons)
 fields: [triplet] [frequency: per thousand] ([number])

GUC 30.1(497)	GCC 54.3(898)	GAC 45.6(754)	GGC 42.6(704)
GUA 4.1(67)	GCA 12.5(206)	GAA 16.5(273)	GGA 7.3(120)
GUG 37.6(621)	GCG 41.7(689)	GAG 32.7(541)	GGG 16.7(276)

Coding GC 64.82% 1st letter GC 65.36% 2nd letter GC 48.07% 3rd letter GC 81.04%

[0223]

TABLE 2F

Codon Usage Table for <i>Shigella flexneri</i> (from www.kazusa.or.jp/codon/). <i>Shigella flexneri</i> [gbdb]: 706 CDS's (180312 codons) fields: [triplet] [frequency: per thousand] [(number)]			
UUU 25.8(4658)	UCU 16.6(2986)	UAU 21.9(3945)	UGU 6.9(1252)
UUC 15.1(2714)	UCC 9.5(1717)	UAC 11.0(1992)	UGC 5.6(1011)
UUA 20.8(3756)	UCA 15.6(2821)	UAA 2.0(362)	UGA 1.4(254)
UUG 13.4(2424)	UCG 6.9(1241)	UAG 0.5(91)	UGG 13.1(2357)
CUU 17.6(3169)	CCU 9.2(1656)	CAU 15.1(2725)	CGU 15.0(2707)
GUC 10.4(1878)	CCC 5.9(1072)	CAC 8.2(1472)	CGC 12.6(2269)
CUA 7.2(1295)	CCA 9.7(1744)	CAA 15.9(2861)	CGA 5.8(1046)
CUG 33.5(6045)	CCG 12.2(2199)	CAG 23.6(4255)	CGG 9.0(1627)
AUU 30.0(5417)	ACU 13.8(2480)	AAU 33.5(6044)	AGU 15.3(2764)
AUC 16.7(3018)	ACC 13.4(2413)	AAC 18.6(3348)	AGC 12.7(2281)
AUA 18.9(3402)	ACA 16.2(2930)	AAA 41.6(7507)	AGA 10.3(1865)
AUG 23.3(4198)	ACG 10.0(1809)	AAG 16.4(2961)	AGG 5.7(1029)
GUU 19.8(3576)	GCU 19.6(3527)	GAU 34.0(6123)	GGU 19.2(3468)
GUC 11.8(2126)	GCC 18.5(3338)	GAC 16.3(2939)	GGC 15.3(2754)
GUA 13.1(2370)	GCA 22.2(4009)	GAA 37.5(6763)	GGA 15.1(2727)
GUG 16.1(2910)	GCG 15.2(2732)	GAG 21.7(3913)	GGG 10.9(1970)

Coding GC 44.63% 1st letter GC 51.72% 2nd letter GC 38.85% 3rd letter GC 43.32%

[0224] In some embodiments of the invention, at least about 10%, at least about, 25%, at least about 50%, or at least about 75% of the codons in a codon-optimized coding sequence are the most preferred codon for that amino acid used in the target organism. In other embodiments, 100% of the codons in the codon-optimized coding sequence are the most preferred codon for that amino acid in the target organism (i.e., the sequence is "fully codon-optimized"). For instance, in the Examples shown below, all of the codons of the sequences characterized as codon-optimized were the most frequently used codons for the target organism; however, any codon substitution that results in a more frequently used codon than the original (native) sequence can be considered "codon-optimized". Table 3, below shows the optimal codon usage in *Listeria monocytogenes* for each amino acid.

TABLE 3

Optimal Codon Usage Table in <i>Listeria monocytogenes</i> .		
Amino Acid	One Letter Code	Optimal <i>Listeria</i> Codon
Alanine	A	GCA
Arginine	R	CGU
Asparagine	N	AAU
Aspartate	D	GAU
Cysteine	C	UGU
Glutamine	Q	CAA
Glutamate	E	GAA
Glycine	G	GGU
Histidine	H	CAU
Isoleucine	I	AUU
Leucine	L	UUA
Lysine	K	AAA
Methionine	M	AUG
Phenylalanine	F	UUU
Proline	P	CCA
Serine	S	AGU
Threonine	T	ACA
Tryptophan	W	UGG
Tyrosine	Y	UAU
Valine	V	GUU

[0225] In some embodiments, the codon-optimized polynucleotides encode a signal peptide. In some embodiments,

the signal peptide is foreign to the bacterium for which the sequence is codon-optimized. In other embodiments, the signal peptide is native to the bacterium for which the sequence is codon-optimized. For instance, in some embodiments, the codon-optimized polynucleotide encodes a signal peptide selected from the group consisting of LLO signal peptide from *Listeria monocytogenes*, Usp45 signal peptide from *Lactococcus lactis*, Protective Antigen signal peptide from *Bacillus anthracis*, p60 signal peptide from *Listeria monocytogenes* and PhoD signal peptide from *B. subtilis* Tat signal peptide. In some embodiments, the codon-optimized polynucleotide encodes a signal peptide other than Protective Antigen signal peptide from *Bacillus anthracis*. In some embodiments, the polynucleotide encoding a signal peptide is codon-optimized for expression in *Listeria monocytogenes*.

[0226] In some embodiments, the codon-optimized polynucleotide encodes a (non-signal peptide) protein that is foreign to the bacterium for which the polynucleotide sequence has been codon-optimized. In some embodiments, the codon-optimized polynucleotide encodes a polypeptide comprising an antigen. For instance, in some embodiments, the codon-optimized polynucleotide encodes a polypeptide comprising an antigen that is a tumor-associated antigen or an antigen that is derived from a tumor-associated antigen.

[0227] In some embodiments, codon-optimization of a polynucleotide encoding a signal peptide and/or other polypeptide enhances expression of a polypeptide (such as a fusion protein, protein chimera and/or a foreign polypeptide encoded by a recombinant nucleic acid molecule, expression cassette, or expression vector) comprising the signal peptide and/or other polypeptide in a bacterium, relative to the corresponding polynucleotide without codon-optimization. In some embodiments, the codon-optimization of the polynucleotide enhances expression by at least about 2-fold, by at least about 5-fold, by at least about 10-fold, or by at least about 20 fold (relative to the corresponding polynucleotide without codon-optimization). In some embodiments, codon-optimization of a polynucleotide encoding a signal peptide and/or other polypeptide enhances secretion of a polypeptide (such as a fusion protein, protein chimera and/or a foreign

polypeptide) comprising the signal peptide and/or other polypeptide from a bacterium, relative to the corresponding polynucleotide without codon-optimization. In some embodiments, the codon-optimization enhances secretion by at least about 2-fold, by at least about 5-fold, by at least about 10-fold, or by at least about 20 fold (relative to the corresponding polynucleotide without codon-optimization). In some embodiments, both the level of expression and secretion is enhanced. Levels of expression and/or secretion can be readily assessed using techniques standard to those in the art such as Western blots of the various relevant bacterial culture fractions.

[0228] VI. Expression cassettes

[0229] Expression cassettes are also provided by the present invention. For instance, in some embodiments, the invention provides an expression cassette comprising any of the recombinant nucleic acid molecules described herein and further comprising promoter sequences operably linked to the coding sequences in the recombinant nucleic acid molecules (e.g., the first polynucleotide encoding a signal peptide and the second polynucleotide encoding the other polypeptide). In some embodiments, the expression cassette is isolated. In some other embodiments, the expression cassette is contained within an expression vector, which may be isolated or may be contained within a bacterium. In still further embodiments, the expression cassette is positioned in the chromosomal DNA of a bacterium. For instance, in some embodiments, the expression cassette has been integrated within the genome of a bacterium. In some embodiments, an expression cassette that is integrated within the genome of a bacterium comprises one or more elements from the genomic DNA. For instance, in some embodiments, a recombinant nucleic acid molecule is inserted in a site in the genomic DNA of a bacterium (e.g., via site-specific integration or homologous recombination) such that the recombinant nucleic acid is operably linked to a promoter already present in the genomic DNA, thereby generating a new expression cassette integrated within the genomic DNA. In some other embodiments, the expression cassette is integrated into the genomic DNA (e.g., via site-specific integration or homologous recombination) as an intact unit comprising both the promoter and the recombinant nucleic acid molecule.

[0230] In some embodiments, the expression cassettes are designed for expression of polypeptides in bacteria. In some embodiments, the expression cassettes are designed for the expression of heterologous polypeptides, such as heterologous antigens in bacteria. In some embodiments, the expression cassettes provide enhanced expression and/or secretion of the polypeptides.

[0231] Generally, an expression cassette comprises the following ordered elements: (1) a promoter and (2) a polynucleotide encoding a polypeptide. In some embodiments, an expression cassette comprises the following elements: (1) a promoter; (2) a polynucleotide encoding a signal peptide; and (3) a polynucleotide encoding a polypeptide (e.g., a heterologous protein). In still other embodiments, an expression cassette comprises the following elements: (1) prokaryotic promoter; (2) Shine-Dalgarno sequence; (3) a polynucleotide encoding a signal peptide; and, (4) a polynucleotide encoding a polypeptide (such as a heterologous protein). In some embodiments, an expression cassette comprises more than one promoter.

[0232] In some embodiments, the expression cassette may also contain a transcription termination sequence inserted downstream from the C-terminus of the translational stop codon related to the heterologous polypeptide. For instance, in some embodiments, a transcription termination sequence may be used in constructs designed for stable integration within the bacterial chromosome. While not required, inclusion of a transcription termination sequence as the final ordered element in a heterologous gene expression cassette may prevent polar effects on the regulation of expression of adjacent genes due to read-through transcription. Accordingly, in some embodiments, appropriate sequence elements known to those who are skilled in the art that promote either rho-dependent or rho-independent transcription termination can be placed in the heterologous protein expression cassette.

[0233] In one aspect, the invention provides an expression cassette comprising the following: (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium; (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the polypeptide.

[0234] In another aspect, the invention provides an expression cassette comprising (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a promoter operably linked to the first and second polynucleotides of the expression cassette, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the second polynucleotide is heterologous to the first polynucleotide. In some embodiments, the polypeptide is heterologous to the bacterium to which the signal peptide is native (i.e., foreign to the bacterium). In some embodiments, the bacterium from which the signal peptide is derived is an intracellular bacterium. In some embodiments, the bacterium is selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria and *E. coli*. In some embodiments the bacterium is a *Listeria* bacterium (e.g., *Listeria monocytogenes*). In some embodiments, the second polynucleotide is codon-optimized for expression in the bacterium.

[0235] In another aspect, the invention provides an expression cassette, wherein the expression cassette comprises (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a *Listeria* bacterium, (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a promoter operably linked to the first and second polynucleotides of the expression cassette, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the expression cassette is a polycistronic

expression cassette. In some embodiments, the second polynucleotide is codon-optimized for expression in the *Listeria* bacterium. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the *Listeria* bacterium (i.e., heterologous to the *Listeria* bacterium). In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the expression cassette comprises more than one promoter.

[0236] In another aspect, the invention provides an expression cassette comprising (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; (b) a second polynucleotide encoding a polypeptide in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the first polynucleotide and/or the second polynucleotide is codon-optimized for expression in a bacterium, such as *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria or *E. coli*. In some embodiments, the polynucleotide(s) is codon-optimized for expression in *Listeria*, such as *Listeria monocytogenes*. In some embodiments, the signal peptide encoded by the codon-optimized first polynucleotide is native to the bacterium for which it is codon-optimized. In some embodiments, the first polynucleotide encoding the signal peptide is heterologous to the second polynucleotide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the expression cassette is a polycistronic expression cassette. In some embodiments, the first polynucleotide, the second polynucleotide, or both the first and second polynucleotide is codon-optimized for expression in a *Listeria* bacterium (e.g., *Listeria monocytogenes*). In some embodiments, the first and second polynucleotides are heterologous to each other. In some embodiments, the polypeptide encoded by the second polynucleotide and the signal peptide are heterologous to each other. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the *Listeria* bacterium (i.e., heterologous to the *Listeria* bacterium). In some embodiments, the expression cassette comprises more than one promoter.

[0237] The invention also provides an expression cassette comprising the following: (a) a polynucleotide encoding a polypeptide foreign to *Listeria*, wherein the polynucleotide is codon-optimized for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the foreign polypeptide. In some embodiments, the polypeptide that is encoded by the expression cassette is an antigen (e.g., see description of some possible antigens above). In some embodiments, the expression cassette further comprises a polynucleotide encoding a signal peptide. The polynucleotide encoding the signal peptide is also operably linked with the promoter so that the expression cassette expresses a fusion protein comprising both the foreign polypeptide and the signal peptide. Polynucleotides encoding signal peptides suitable for use in the expression cassette include, but are not limited to, those described above. In some embodiments, the polynucleotide encoding a signal peptide that is included in the expression cassette is codon-optimized for expression in a bacterium such as *Listeria* (e.g., a *L. monocytogenes* bacterium) as described above.

[0238] The invention also provides an expression cassette comprising the following: (a) a first polynucleotide encoding a non-*Listeria* signal peptide; (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to both the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising both the non-*Listeria* signal peptide and the polypeptide. In some embodiments, the expression cassette is a polycistronic expression cassette. In some embodiments, the first polynucleotide, the second polynucleotide, or both the first and second polynucleotide is codon-optimized for expression in *Listeria* (e.g., *Listeria monocytogenes*). In some embodiments, the first and second polynucleotides are heterologous to each other. In some embodiments, the polypeptide encoded by the second polynucleotide and the signal peptide are heterologous to each other. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the *Listeria* bacterium (i.e., heterologous to the *Listeria* bacterium). In some embodiments, the expression cassette comprises more than one promoter.

[0239] The invention further provides an expression cassette, wherein the expression cassette comprises (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes a protein chimera comprising the polypeptide encoded by the second polynucleotide and the autolysin, or catalytically active fragment or catalytically active variant thereof, wherein in the protein chimera the polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof, or is inserted within the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the protein chimera is catalytically active as an autolysin. In some embodiments, the polypeptide is heterologous to the autolysin. In some embodiments, the bacterial autolysin is from an intracellular bacterium (e.g., *Listeria*). In some embodiments, the second polynucleotide encoding the polypeptide is inserted within the first polynucleotide encoding the autolysin, or catalytically active fragment or catalytically active variant thereof, and the expression cassette encodes a protein chimera in which the polypeptide is inserted within the autolysin, or catalytically active fragment or catalytically active variant thereof (i.e., the polypeptide is embedded within the autolysin or catalytically active fragment or catalytically active variant thereof). In alternative embodiments, the second polynucleotide is positioned outside of the first polynucleotide encoding the autolysin, or catalytically active fragment or catalytically active variant thereof, and the expression cassette encodes a protein chimera in which the polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the polypeptide is heterologous to the autolysin. In some embodiments, the first polynucleotide and the second polynucleotide are heterologous to each other. In some embodiments, the autolysin is a SecA2-dependent autolysin. In some embodiments, the autolysin is a peptidoglycan hydrolase (e.g., N-acetylmuramidase or p60). In some embodiments, the expression cassette further comprises a poly-

nucleotide encoding a signal peptide (e.g., a signal peptide normally associated with the autolysin or a signal peptide heterologous to the signal peptide). For instance, in some embodiments, the expression cassette encodes a protein chimera comprising a p60 signal peptide, the p60 protein (or catalytically active fragment or catalytically active variant thereof), and a polypeptide heterologous to p60, embedded within the p60 sequence. In some embodiments, the polypeptide encoded by the second polynucleotide is a non-Listerial polypeptide.

[0240] In another aspect, the invention provides an expression cassette comprising (a) a first polynucleotide encoding a signal peptide, (b) a second polynucleotide encoding a secreted protein, or a fragment thereof, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, (c) a third polynucleotide encoding a polypeptide heterologous to the secreted protein, or fragment thereof, wherein the third polynucleotide is in the same translational reading frame as the first and second polynucleotides, and (d) promoter operably linked to the first, second, and third polynucleotides, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the second polynucleotide, and the secreted protein, or fragment thereof, and wherein the polypeptide encoded by the third polynucleotide is fused to the secreted protein, or fragment thereof, or is positioned within the secreted protein, or fragment thereof, in the protein chimera.

[0241] In some embodiments, the promoters in the expression cassettes described herein (or recombinant nucleic acid molecules described herein) are prokaryotic promoters. For instance, the prokaryotic promoters can be Listerial promoters. In some embodiments, the Listerial promoter is an hly promoter. In some embodiments, the promoters are prfA-dependent promoters (e.g., an actA promoter). In some embodiments, the promoters are constitutive promoters (e.g., ap60 promoter). In some embodiments, the expression cassette comprising a recombinant nucleic acid molecule described herein comprises an hly, actA, or p60 promoter operably linked to the polynucleotides of the recombinant nucleic acid molecule. One of ordinary skill in the art will be readily able to identify additional prokaryotic and/or Listerial promoters suitable for use in the expression cassettes in view of the intended use of the expression cassette and host bacteria into which the expression cassette will be placed.

[0242] For instance, a variety of mycobacterial promoters suitable for use in the recombinant expression cassettes within mycobacteria and other bacteria are known. These include the *Mycobacterium bovis* BCG promoters HSP60 and HSP70, and also include such promoters as the mycobactin promoters, α -antigen promoter and 45 kDa antigen promoter of *M. tuberculosis* and BCG, the superoxide dismutase promoter, MBP-70, the mycobacterial asd promoter, the mycobacterial 14 kDa and 12 kDa antigen promoters, mycobacteriophage promoters such as the Bxb1, Bxb2, and Bxb3 promoters, the L1 and L5 promoters, the D29 promoter and the TM4 promoters (see, e.g., U.S. Pat. No. 6,566,121). Promoters suitable for use in *Bacillus anthracis* include, but are not limited to, the pagA promoter, the alpha-amylase promoter (Pamy), and Pntr (see, e.g., Gat et al., *Infect. Immun.*, 71:801-13 (2003)). Promoters suitable for use in recombinant *Salmonella* expression cassettes and

vaccines are also known and include the nirB promoter, the osmC promoter, P(pagC), and P(tac) (see, e.g., Bumann, *Infect. Immun.* 69:7493-500 (2001); Wang et al., *Vaccine*, 17:1-12 (1999); McSorley et al., *Infect. Immun.* 65:171-8 (1997)). A variety of *E. coli* promoters are also known to those of ordinary skill in the art.

[0243] In some embodiments, the promoter used in an expression cassette described herein is a constitutive promoter. In other embodiments, the promoter used in an expression cassettes described herein is an inducible promoter. The inducible promoter can be induced by a molecule (e.g., a protein) endogenous to the bacteria in which the expression cassette is to be used. Alternatively, the inducible promoter can be induced by a molecule (e.g. a small molecule or protein) heterologous to the bacteria in which the expression cassette is to be used. A variety of inducible promoters are well-known to those of ordinary skill in the art.

[0244] In some embodiments of the expression cassettes, at the 3'-end of the promoter is a poly-purine Shine-Dalgarno sequence, the element required for engagement of the 30S ribosomal subunit (via 16S rRNA) to the heterologous gene RNA transcript and initiation of translation. The Shine-Dalgarno sequence has typically the following consensus sequence: 5'-NAGGAGGU-N₅₋₁₀-AUG (start codon)-3' (SEQ ID NO:85). There are variations of the poly-purine Shine-Dalgarno sequence. Notably, the *Listeria* hly gene that encodes listeriolysin O (LLO) has the following Shine-Dalgarno sequence: AAGGAGAGTGAAACCCATG' (SEQ ID NO:70) (Shine-Dalgarno sequence is underlined, and the translation start codon is bolded).

[0245] The construction of expression cassettes for use in bacteria, and even the construction of expression cassettes specifically for use in recombinant bacterial vaccines, are known in the art. For instance, descriptions of the production and use of a variety of bacterial expression cassettes and/or recombinant bacterial vaccines can be found in the following references, each of which is hereby incorporated by reference herein in its entirety: Horwitz et al., *Proc. Natl. Acad. Sci. USA*, 97:13853-8 (2000); Garmory et al., *J. Drug Target*, 11:471-9 (2003); Kang et al., *FEMS Immunol. Med. Microbiol.*, 37:99-104 (2003); Garmory et al., *Vaccine*, 21:3051-7 (2003); Kang et al., *Infect. Immun.*, 1739-49 (2002); Russman, et al., *J. Immunol.*, 167:357-65 (2001); Harth et al., *Microbiology*, 150:2143-51 (2004); Varaldo et al., *Infect. Immun.*, 72:3336-43 (2004); Goonetilleke et al., *J. Immunol.*, 171:1602-9 (2003); Uno-Furuta et al., *Vaccine*, 21:3149-56 (2003); Biet et al., *Infect. Immun.*, 71:2933-7 (2003); Bao et al., *Infect. Immun.*, 71:1656-61 (2003); Kawahara et al., *Clin. Immunol.*, 105:326-31 (2002); Anderson et al., *Vaccine*, 18:2193-202 (2000); Bumann, *Infect. Immun.*, 69:7493-500 (2001); Wang et al., *Vaccine*, 17:1-12 (1999); McSorley et al., *Infect. Immun.*, 65:171-8 (1997); Gat et al., *Infect. Immun.*, 71:801-13 (2003); U.S. Pat. No. 5,504,005; U.S. Pat. No. 5,830,702; U.S. Pat. No. 6,051,237; U.S. patent Publication No. 2002/0025323; U.S. patent Publication No. 2003/0202985; WO 04/062597; U.S. Pat. No. 6,566,121; and U.S. Pat. No. 6,270,776.

[0246] In some embodiments, it is desirable to construct expression cassettes that utilize bicistronic, polycistronic (also known as multicistronic) expression of heterologous coding sequences. Such expression cassettes can utilize, for

example, a single promoter that is operably linked to two or more independent coding sequences. These coding sequences can, for example, correspond to individual genes or can, alternatively, correspond to desired and/or selected sub-fragments of a whole designated gene. In this later example, a gene might contain a sequence encoding a hydrophobic trans-membrane domain, which may potentially inhibit efficient secretion from *Listeria*. Thus, it may be desirable to segregate in two sub-fragments the coding sequence of this gene from the hydrophobic domain; in this instance the two sub-fragments are then expressed as a bicistronic message. Utilization of polycistronic expression requires that the 30s ribosome subunit stay on the polycistronic RNA message following translation termination of the first coding sequence and release of the 50s ribosome sub-unit, and subsequently "read-through" the RNA message to the next initiation codon, during which the 50s ribosome sub-unit binds to the RNA-bound 30s ribosome subunit, and re-initiating translation.

[0247] *Listeria monocytogenes*, like other bacteria, utilizes polycistronic expression of its genomic repertoire. By way of example, the sequence of a *Listeria monocytogenes* intergenic region from a selected polycistronic message can be used to construct polycistronic expression cassettes for expression of a selected heterologous protein from recombinant *Listeria* species. For example, several of the prfA-dependent virulence factors from *Listeria monocytogenes* are expressed from polycistronic message. For instance, the *Listeria monocytogenes* ActA and PlcB proteins are expressed as a bicistronic message. The DNA sequence corresponding to the *Listeria monocytogenes* actA-plcB intergenic sequence (5'-3') is shown below

(SEQ ID NO : 71)
5' - TAAACACAGAACGAAAGAAAAGTGAGGTGAATGA - 3'

[0248] (The Shine-Dalgarno sequence for translation initiation of plcB is shown in bold. The first 3 nucleotides of the sequence correspond to an Ochre stop codon.) For a non-limiting example of a bicistronic expression vector, a bicistronic hEphA2 expression vector for use in *Listeria monocytogenes*, see Example 28, below.

[0249] Alternatively, other known intergenic or synthetic sequences can be used to construct polycistronic expression cassettes for use in *Listeria* or other bacteria. Construction of intergenic regions which lead to substantial secondary RNA structure should be prevented, to avoid unwanted transcription termination by a rho-independent mechanism.

[0250] Importantly, if secretion of any or all translated proteins expressed from the polycistronic message is desired, signal peptides must be functionally linked to each coding region. In some embodiments, these signal peptides differ from each other.

[0251] Thus, in some embodiments, the expression cassettes described herein for use in *Listeria* or other bacteria are polycistronic (e.g., bicistronic). Two or more polypeptides are encoded by the bicistronic or polycistronic expression cassettes as discrete polypeptides. In some embodiments, the bicistronic or polycistronic expression cassettes comprise an intergenic sequence (e.g., from a bicistronic or polycistronic gene) positioned between the coding

sequences of the two polypeptides. In some embodiments, the intergenic sequence comprises a sequence which promotes ribosomal entry and initiation of translation. In some embodiments, the intergenic sequence comprises a Shine-Dalgarno sequence. In some embodiments, the intergenic sequence is the *Listeria monocytogenes* actA-plcB intergenic sequence. Typically, the intergenic sequence is positioned between a polynucleotide sequence encoding a first polypeptide (or a first fusion protein comprising a first polypeptide and a signal peptide) and a polynucleotide sequence encoding a second polypeptide (or a second fusion protein comprising a second polypeptide and signal peptide).

[0252] Accordingly, in one aspect, the invention provides an expression cassette comprising the following: (a) a first polynucleotide encoding a first polypeptide; (b) a second polynucleotide encoding a second polypeptide; (c) an intergenic sequence positioned between the first and second polynucleotides; and (f) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes the first and second polypeptides as two discrete polypeptides. In some embodiments, the first and second polypeptides are polypeptides selected from any of the polypeptides described herein (e.g., in Section IV, above). In some embodiments, at least one of the first or second polypeptides comprises an antigen. In some embodiments, the first and second polynucleotides each comprise a (different or the same) fragment of the same antigen. In some embodiments, the antigen is a tumor-associated antigen or is derived from a tumor-associated-antigen.

[0253] The invention further provides an expression cassette comprising the following: (a) a first polynucleotide encoding a first signal peptide; (b) a second polynucleotide encoding a first (non-signal) polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; (c) a third polynucleotide encoding a second signal peptide; (d) a fourth polynucleotide encoding a second (non-signal) polypeptide, wherein the fourth polynucleotide is in the same translational reading frame as the third polynucleotide; (e) an intergenic sequence (typically positioned between the second polynucleotide and the third polynucleotide); and (f) a promoter operably linked to the first polynucleotide, second polynucleotide, third polynucleotide, and fourth polynucleotide, so that the expression cassette encodes both a first fusion protein comprising the first signal peptide and the first polypeptide and a second fusion protein comprising the second signal peptide and second polypeptide. In some embodiments, the one or more of the polynucleotides encoding a signal peptide is codon-optimized for expression in a bacterium. In some embodiments, the third and/or fourth polynucleotides are codon-optimized for expression in a bacterium (preferably in addition to codon-optimization of the polynucleotides encoding the signal peptides). In some embodiments, the first and/or second signal peptide is a non-secA1 bacterial signal peptide. In some embodiments, the intergenic sequence is the *Listeria monocytogenes* actA-plcB intergenic sequence. In some embodiments, the second and third polypeptides are polypeptides selected from any of the polypeptides described herein (e.g., in Section IV, above), such as polypeptides comprising antigens. In some embodiments, the first and second polypeptides are polypeptides selected from any of the polypeptides described herein (e.g., in Section IV, above). In some embodiments, at least one of

the first or second polypeptides comprises an antigen. In some embodiments, the first and second polynucleotides each comprise a fragment of the same antigen. In some embodiments, the antigen is a tumor-associated antigen or is derived from a tumor-associated-antigen.

[0254] For instance, the invention provides a polycistronic expression cassette for expression of heterologous polypeptides in *Listeria*, wherein the expression cassette encodes at least two discrete non-*Listerial* polypeptides. In some embodiments, the polycistronic expression cassette is a bicistronic expression cassette which encodes two discrete non-*Listerial* polypeptides. In some embodiments, the expression cassette comprises the following: (a) a first polynucleotide encoding a first non-*Listerial* polypeptide; (b) a second polynucleotide encoding a second non-*Listerial* polypeptide; (c) an intergenic sequence positioned between the first and second polynucleotides; and (d) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes the first and second polypeptides as two discrete polypeptides. If the expression cassette is a polycistronic expression cassette that encodes three polypeptides as discrete polypeptides, the expression cassette will comprise a third polynucleotide operably linked to the promoter and a second intergenic sequence positioned between the second and third polynucleotide. In some embodiments, at least one of the non-*Listerial* polypeptides comprises an antigen. In some embodiments, at least two of the non-*Listerial* polypeptides each comprises a fragment of the same antigen.

[0255] In some embodiments, the expression cassette comprises the following: (a) a first polynucleotide encoding a first signal peptide; (b) a second polynucleotide encoding a first (non-signal) non-*Listerial* polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; (c) a third polynucleotide encoding a second signal peptide; (d) a fourth polynucleotide encoding a second (non-signal) non-*Listerial* polypeptide, wherein the fourth polynucleotide is in the same translational reading frame as the third polynucleotide; (e) an intergenic sequence positioned between the second polynucleotide and the third polynucleotide; and (f) a promoter operably linked to the first polynucleotide, second polynucleotide, third polynucleotide, and fourth polynucleotide, so that the expression cassette encodes both a first fusion protein comprising the first signal peptide and the first polypeptide and a second fusion protein comprising the second signal peptide and second polypeptide. In some embodiments, at least one of the non-*Listerial* polypeptides is an antigen. In some embodiments, at least two of the non-*Listerial* polypeptides are each fragments of the same antigen.

[0256] The invention also provides a method of using any of the expression cassettes described herein to produce a recombinant bacterium (e.g. a recombinant *Listeria* bacterium). In some embodiments, the method of using an expression cassette described herein to make a recombinant bacterium comprises introducing the expression cassette into a bacterium. In some embodiments, the expression cassette is integrated into the genome of the bacterium. In some other embodiments, the expression cassette is on a plasmid which is incorporated within the bacterium. In some embodiments, incorporation of the expression cassette into the bacterium occurs by conjugation. The introduction of the

expression cassette into the bacterium can be effected by any of the standard techniques known in the art. For instance, incorporation of the expression cassette into the bacterium can occur by conjugation, transduction (transfection), or transformation.

[0257] VII. Vectors

[0258] The invention further provides vectors, such as expression vectors, which comprise any one of the expression cassettes and/or recombinant nucleic acid molecules described herein. In some embodiments, the vector is a plasmid. In some embodiments, the vector is linear. In some embodiments, the vector is circular. In some embodiments, the vector is an integration or homologous recombinant vector. In some embodiments, the vector is pAM401. In some embodiments, the vector is pPL2. In some embodiments, the vector is isolated.

[0259] As indicated above, in some embodiments, an expression cassette described herein is contained within an expression vector. In some embodiments, the vector is a plasmid. In other embodiments the vector is linear. In alternative embodiments, the expression cassette is inserted (i.e. integrated) within genomic DNA of a bacterium using an expression vector. In some embodiments, the expression vector is linear. In other embodiments, the expression vector is circular.

[0260] Expression vectors suitable for use in bacteria such as *Listeria* are known to those skill in the art. There are a variety of suitable vectors suitable for use as a plasmid construct backbone for assembly of the expression cassettes. A particular plasmid construct backbone is selected based on whether expression of the polynucleotide (i.e., a polynucleotide encoding a heterologous antigen) from the bacterial chromosome or from an extra-chromosomal episome is desired.

[0261] In some embodiments, incorporation of the expression cassette (and/or recombinant nucleic acid molecule) into the bacterial chromosome of *Listeria monocytogenes* (*Listeria*) is accomplished with an integration vector that contains an expression cassette for a listeriphage integrase that catalyzes sequence-specific integration of the vector into the *Listeria* chromosome. For example, the integration vectors known as pPL1 and pPL2 program stable single-copy integration of a heterologous protein expression cassette within an innocuous region of the bacterial genome, and have been described in the literature (Lauer et. al. 2002 J. Bacteriol. 184:4177-4178; U.S. patent Publication No. 20030203472). The integration vectors are stable as plasmids in *E. coli* and are introduced via conjugation into the desired *Listeria* background. Each vector lacks a *Listeria*-specific origin of replication and encodes a phage integrase, such that the vectors are stable only upon integration into a chromosomal phage attachment site. Starting with a desired plasmid construct, the process of generating a recombinant *Listeria* strain expressing a desired protein(s) takes approximately one week. The pPL1 and pPL2 integration vectors are based, respectively, on the U153 and PSA listeriphages. The pPL1 vector integrates within the open reading frame of the *comK* gene, while pPL2 integrates within the *tRNA^{Arg}* gene in such a manner that the native sequence of the gene is restored upon successful integration, thus keeping its native expressed function intact. The pPL1 and pPL2 integration vectors contain a multiple cloning site sequence in

order to facilitate construction of plasmids containing a recombinant nucleic acid molecule or an expression cassette such as the heterologous protein expression cassette. Some specific examples of the use of the pPL2 integration vector are described in Example 2 and Example 3, below.

[0262] Alternatively, incorporation of the expression cassette (and/or recombinant nucleic acid molecule) into the *Listeria* chromosome can be accomplished through allelic exchange methods, known to those skilled in the art. In particular, compositions in which it is desired to not incorporate a gene encoding an antibiotic resistance protein as part of the construct containing the expression cassette, methods of allelic exchange are desirable. For example, the pKSV7 vector (Camilli et. al. *Mol. Microbiol.* (1993) 8,143-157), contains a temperature-sensitive *Listeria* Gram-positive replication origin which is exploited to select for recombinant clones at the non-permissive temperature that represent the pKSV7 plasmid recombined into the *Listeria* chromosome. The pKSV7 allelic exchange plasmid vector contains a multiple cloning site sequence in order to facilitate construction of plasmids containing the protein expression cassette, and also a chloramphenicol resistance gene. For insertion into the *Listeria* chromosome, the expression cassette construct may be flanked by approximately 1 kb of chromosomal DNA sequence that corresponds to the precise location of desired integration. The pKSV7-expression cassette plasmid may be introduced into a desired bacterial strain by electroporation, according to standard methods for electroporation of Gram positive bacteria. A non-limiting example of a method of effecting allelic exchange using the pKSV7 vector is provided in Example 16 below.

[0263] In other embodiments, it may be desired to express the polypeptide (including a fusion protein comprising a polypeptide) from a stable plasmid episome. Maintenance of the plasmid episome through passaging for multiple generations requires the co-expression of a protein that confers a selective advantage for the plasmid-containing bacterium. As non-limiting examples, the protein co-expressed from the plasmid in combination with the polypeptide may be an antibiotic resistance protein, for example chloramphenicol, or may be a bacterial protein (that is expressed from the chromosome in wild-type bacteria), that can also confer a selective advantage. Non-limiting examples of bacterial proteins include enzyme required for purine or amino acid biosynthesis (selected using defined media lacking relevant amino acids or other necessary precursor macromolecules), or a transcription factor required for the expression of genes that confer a selective advantage in vitro or in vivo (Gunn et. al. 2001 *J. Immunol.* 167:6471-6479). As a non-limiting example, pAM401 is a suitable plasmid for episomal expression of a selected polypeptide in diverse Gram-positive bacterial genera (Wirth et. al. 1986 *J. Bacteriol.* 165:831-836). For further description of exemplary uses of pAM401, see Examples 3 and 13, below.

[0264] Incorporation of the expression cassette into the bacterial chromosome of *B. anthracis* can, for instance, be accomplished with an integration vector that contains an expression cassette for a phage integrase that catalyzes sequence-specific integration of the vector into the *B. anthracis* chromosome. The integrase and attachment site of a *B. anthracis* phage can be used to derive an integration vector, to incorporate desired antigen expression cassettes into the vaccine composition. As a non-limiting example, the

integrase and attachment site from the *B. anthracis* temperate phage w-alpha is used to derive a *B. anthracis* specific integration vector (McCloy, E. W. 1951. Studies on a lysogenic *Bacillus* strain. I. A bacteriophage specific for *Bacillus anthracis*. *J. Hyg.* 49:114-125).

[0265] Alternatively, incorporation of an antigen expression cassette into the *B. anthracis* chromosome can be accomplished through allelic exchange methods, known to those skilled in the art. See, e.g., Gat et al., *Infect. Immun.*, 71:801-13 (2003). In particular, compositions in which it is desired to not incorporate a gene encoding an antibiotic resistance protein as part of the construct containing the expression cassette, methods of allelic exchange are desirable. For example, the pKSV7 vector (Camilli et. al. *Mol. Microbiol.* 1993 8,143-157), contains a temperature-sensitive *Listeria*-derived Gram positive replication origin which is exploited to select for recombinant clones at the non-permissive temperature that represent the pKSV7 plasmid recombined into the bacterial chromosome. The pKSV7 allelic exchange plasmid vector contains a multiple cloning site sequence in order to facilitate construction of plasmids containing the expression cassette, and also a chloramphenicol resistance gene. For insertion into the *Bacillus anthracis* chromosome, the expression cassette construct may be flanked by approximately 1 kb of chromosomal DNA sequence that corresponds to the precise location of desired integration. The pKSV7-expression cassette plasmid may be introduced into a desired bacterial strain by electroporation, according to standard methods for electroporation of Gram positive bacteria. A non-limiting example of a method of effecting allelic exchange in *B. anthracis* is provided in U.S. patent application Ser. No. 10/883,599, incorporated by reference herein in its entirety. In particular, allelic exchange using the pKSV7 vector can be used in strains of *B. anthracis* to add a desired antigen expression cassette at any desired location within the bacterial chromosome.

[0266] The allelic exchange methods described above using pKSV7 are broadly applicable to use in gram positive bacteria. In addition, a variety of expression vectors useful in bacteria, including recombinant bacterial vectors, are known to those of ordinary skill in the art. Examples include those vectors described in the following references, each of which is incorporated by reference herein in its entirety: Horwitz et al., *Proc. Natl. Acad. Sci. USA*, 97:13853-8 (2000); Garmory et al., *J. Drug Target*, 11:471-9 (2003); Kang et al., *FEMS Immunol. Med. Microbiol.*, 37:99-104 (2003); Garmory et al., *Vaccine*, 21:3051-7 (2003); Kang et al., *Infect. Immun.*, 1739-49 (2002); Russman, et al., *J. Immunol.*, 167:357-65 (2001); Harth et al., *Microbiology*, 150:2143-51 (2004); Valardo et al., *Infect. Immun.*, 72:3336-43 (2004); Goonetilleke et al., *J. Immunol.*, 171:1602-9 (2003); Uno-Furuta et al., *Vaccine*, 21:3149-56 (2003); Biet et al., *Infect. Immun.*, 71:2933-7 (2003); Bao et al., *Infect. Immun.*, 71:1656-61 (2003); Kawahara et al., *Clin. Immunol.*, 105:326-31 (2002); Anderson et al., *Vaccine*, 18:2193-202 (2000); Bumann, *Infect. Immun.*, 69:7493-500 (2001); Wang et al., *Vaccine*, 17:1-12 (1999); McSorley et al., *Infect. Immun.*, 65:171-8 (1997); Gat et al., *Infect. Immun.*, 71:801-13 (2003); U.S. Pat. No. 5,504,005; U.S. Pat. No. 5,830,702; U.S. Pat. No. 6,051,237; U.S. patent Publication No. 2002/0025323; U.S. patent Publication No. 2003/0202985; WO 04/062597; U.S. Pat. No. 6,566,121; and U.S. Pat. No. 6,270,776.

[0267] The invention further provides expression vectors comprising expression cassettes comprising the following: (a) a first polynucleotide encoding a first signal peptide; (b) a second polynucleotide encoding a first polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; (c) an intergenic sequence; (d) a third polynucleotide encoding a second signal peptide; (e) a fourth polynucleotide encoding a second polypeptide, wherein the fourth polynucleotide is in the same translational reading frame as the third polynucleotide; and (f) a promoter operably linked to the first polynucleotide, second polynucleotide, third polynucleotide, fourth polynucleotide, and intergenic sequence, such that the expression cassette encodes both a first fusion protein comprising the first signal peptide and the first polypeptide and a second fusion protein comprising the second signal peptide and second polypeptide.

[0268] The invention further provides methods of using any of the expression vectors described herein to produce a recombinant bacterium (e.g. a recombinant *Listeria* bacterium). In some embodiments, the method of using an expression vector described herein to make a recombinant bacterium comprises introducing the expression vector into a bacterium.

[0269] VIII. Bacteria and other Host Cells

[0270] The invention further provides host cells comprising the recombinant nucleic acid molecules, expression cassettes, and/or vectors described herein (see, e.g., the Summary of the Invention and Sections I, II, VI, and VII of the Detailed Description, above, as well as the specific Examples below). In some embodiments, the cells are prokaryotic. In some embodiments, the cells are eukaryotic. In some embodiments, the cells are mammalian. In some embodiments, the cells are antigen-presenting cells, such as dendritic cells. In some embodiments, the cells are bacterial cells. In some embodiments, the host cells are isolated.

[0271] For example, the invention provides bacteria comprising the recombinant nucleic acid molecules, expression cassettes, and/or the vectors described herein (see, e.g., Summary of the Invention and Sections I, II, VI, and VII of the Detailed Description, above, as well as the specific examples below). The bacteria comprising these polynucleotides are alternatively referred to herein as "recombinant bacteria," and a bacterium comprising a recombinant nucleic acid molecule, expression cassette, or vector described herein is alternatively referred to herein as "a recombinant bacterium." In some embodiments, the bacteria comprising the recombinant nucleic acid molecules, expression cassettes, and/or expression vectors are isolated. In some embodiments, the recombinant bacteria comprising the recombinant nucleic acid molecules, expression cassettes, and/or expression vectors express the polypeptides or fusion proteins encoded by the recombinant nucleic acid molecules, expression cassettes, and/or expression vectors contained therein. In some embodiments, the recombinant bacteria secrete the polypeptides or fusion proteins encoded by the recombinant nucleic acid molecules, expression cassettes, and/or expression vectors contained therein. In some embodiments, the recombinant bacteria express and secrete the polypeptides and/or fusion proteins in an amount sufficient to generate an immune response in a host upon administration of the bacteria (or a composition comprising the bacteria) to a host (e.g., a human subject).

[0272] In some embodiments, the bacteria are selected from the group consisting of gram positive bacteria, Gram negative bacteria, intracellular bacteria and mycobacteria. In some embodiments, the bacteria are gram positive bacteria. In some embodiments of the invention, the bacteria are intracellular bacteria (e.g., facultative intracellular bacteria). In some embodiments the bacteria belong to the genus *Listeria*. In other embodiments, the bacteria are members of the species *Listeria monocytogenes*. In some other embodiments the bacteria are members of the *Listeria ivanovii*, *Listeria seeligeri*, or *Listeria innocua* species. In some embodiments, the bacteria are members of the genus *Bacillus*. In another embodiment, the bacteria are *Bacillus anthracis*. In still another embodiment, the bacteria are *Yersinia pestis*. In other embodiments of the invention, the bacteria are from the genus *Salmonella*. In some embodiments, the bacteria are *Salmonella typhimurium*. In some embodiments, the bacteria belong to the genus *Shigella*. For instance, in some embodiments, the bacteria are *Shigella flexneri*. In some embodiments, the bacteria are members of the genus *Brucella*. In an alternative embodiment, the bacteria are mycobacteria. The mycobacteria is optionally a member of the species *Mycobacterium tuberculosis*. In some embodiments, the bacteria are *Bacillus Calmette-Guérin* (BCG). In some embodiments, the bacteria are *E. coli*, for instance, an *E. coli* which has been modified to express Listeriolysin O (LLO). Accordingly, in some embodiments, the bacteria comprising the recombinant nucleic acid molecules, expression cassettes, and/or vectors described herein are selected from the group consisting of *Listeria*, *Bacillus anthracis*, *Yersinia pestis*, *Salmonella*, and mycobacteria. In some other embodiments, the bacteria comprising the recombinant nucleic acid molecules, expression cassettes, and/or vectors described herein are selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria and *E. coli*.

[0273] In some embodiments, the bacteria of the invention that are modified through the insertion of the recombinant nucleic acid molecules, expression cassettes, and/or vectors described herein (e.g., see the Summary of the Invention, Sections I, II, VI, and VII of the Detailed Description, above, and the Examples, below) to express polypeptides, and, in at least some embodiments, secrete the polypeptides, are wild-type bacteria. For instance, in some embodiments, the recombinant bacterium is a wild-type *Listeria* bacterium, such as a *Listeria monocytogenes* bacterium, which comprises the recombinant nucleic acid molecule, expression cassette, and/or vector. However, in some embodiments of the invention, the bacteria comprising the expression cassettes and/or vectors is a mutant strain of the bacteria. In some embodiments, the bacteria are attenuated. In some embodiments, the bacteria are an attenuated mutant strain of bacteria. A mutant in which a gene "xyz" has been deleted is alternatively referred to herein as Δxyz or xyz or an xyz deletion mutant. For instance, a bacterial strain in which the *uvrA* gene has been deleted is alternatively referred to herein as *uvrA* mutant, $\Delta uvrA$, or *uvrA*⁻. In addition, it will be understood by one of ordinary skill in the art that a reference to a particular mutant or strain as an "xyz" mutant or "xyz" strain will sometimes refer to a mutant or strain in which the xyz gene has been deleted.

[0274] The mutation in a mutant bacterium comprising the expression cassettes and/or expression vectors may be a mutation of any type. For instance, the mutation may

constitute a point mutation, a frame-shift mutation, an insertion, a deletion of part or all of a gene. In addition, in some embodiments of the modified strains, a portion of the bacterial genome has been replaced with a heterologous polynucleotide. In some embodiments, the mutations are naturally-occurring. In other embodiments, the mutations are the results of artificial mutation pressure. In still other embodiments, the mutations in the bacterial genome are the result of genetic engineering.

[0275] In some embodiments, the bacteria comprising any one of the recombinant nucleic acid molecules, expression cassettes and/or vectors described herein are attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation (relative to the wild-type bacteria). The bacteria may be attenuated by mutation or by other modifications. In some embodiments, the bacteria comprising any one of the recombinant nucleic acid molecules, expression cassettes and/or expression vectors described herein are attenuated for cell-to-cell spread (e.g., *Listeria monocytogenes* actA mutants). In some embodiments, the bacteria comprising any one of the recombinant nucleic acid molecules, expression cassettes and/or expression vectors described herein are attenuated for entry into non-phagocytic cells (e.g., *Listeria monocytogenes* internalin mutants, such as inlB deletion mutants). In some embodiments, the bacteria comprising any one of the recombinant nucleic acid molecules, expression cassettes and/or expression vectors described herein are attenuated for proliferation. In some embodiments, the bacteria are attenuated both for cell-to-cell spread and for entry into non-phagocytic cells.

[0276] In some embodiments, the bacteria comprising the expression cassettes and/or expression vectors described herein are attenuated for cell-to-cell spread. In some embodiments, the bacteria (e.g., *Listeria*) are defective with respect to ActA (relative to the non-mutant or wildtype bacteria), or its equivalent (depending on the organism). In some embodiments, the bacteria comprise one or more mutation in actA. For instance, the bacterium (e.g., *Listeria*) may be an actA deletion mutant. ActA is the actin polymerase encoded by the actA gene (Kocks, et al., *Cell*, 68:521-531 (1992); Genbank accession no. AL591974, nts 9456-11389). The actin polymerase protein is involved in the recruitment and polymerization of host F-actin at one pole of the *Listeria* bacterium. Subsequent polymerization and dissolution of actin results in *Listeria* propulsion throughout the cytosol and into neighboring cells. This mobility enables the bacteria to spread directly from cell-to-cell without further exposure to the extracellular environment, thus escaping host defenses such as antibody development. In some embodiments, the attenuated *Listeria* optionally comprises both a mutation in an internalin gene, such as inlB, and in actA. The *Listeria* strain of this embodiment of the present invention is attenuated for entry into non-phagocytic cells as well as attenuated for cell-to-cell spreading.

[0277] In some embodiments, the capacity of the attenuated bacterium for cell-to-cell spread is reduced by at least about 10%, at least about 25%, at least about 50%, at least about 75%, or at least about 90%, relative to a bacterium without the attenuating mutation (e.g., the wild type bacterium). In some embodiments, the capacity of the attenuated bacterium for cell-to-cell spread is reduced by at least about 25% relative to a bacterium without the attenuating muta-

tion. In some embodiments, the capacity of the attenuated bacterium attenuated for cell-to-cell spread is reduced by at least about 50% relative to a bacterium without the attenuating mutation.

[0278] In vitro assays for determining whether or not a bacterium such as a *Listeria* bacterium is attenuated for cell-to-cell spread are known to those of ordinary skill in the art. For example, the diameter of plaques formed over a time course after infection of selected cultured cell monolayers can be measured. Plaque assays within L2 cell monolayers can be performed as described previously in Sun, A., A. Camilli, and D. A. Portnoy. 1990, Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect. Immun.* 58:3770-3778, with modifications to the methods of measurement, as described by in Skoble, J., D. A. Portnoy, and M. D. Welch. 2000, Three regions within ActA promote Arp2/3 complex-mediated actin nucleation and *Listeria monocytogenes* motility. *J. Cell Biol.* 150:527-538. In brief, L2 cells are grown to confluency in six-well tissue culture dishes and then infected with bacteria for 1 h. Following infection, the cells are overlaid with media warmed to 40° C. that is comprised of DME containing 0.8% agarose, Fetal Bovine Serum (e.g., 2%), and a desired concentration of Gentamicin. The concentration of Gentamicin in the media dramatically affects plaque size, and is a measure of the ability of a selected *Listeria* strain to effect cell-to-cell spread (Glomski, I J., M. M. Gedde, A. W. Tsang, J. A. Swanson, and D. A. Portnoy. 2002. *J. Cell Biol.* 156:1029-1038). For example, at 3 days following infection of the monolayer the plaque size of *Listeria* strains having a phenotype of defective cell-to-cell spread is reduced by at least 50% as compared to wild-type *Listeria*, when overlaid with media containing Gentamicin at a concentration of 50 µg/ml. On the other hand, the plaque size between *Listeria* strains having a phenotype of defective cell-to-cell spread and wild-type *Listeria* is similar, when infected monolayers are overlaid with media+agarose containing only 5 µg/ml gentamicin. Thus, the relative ability of a selected strain to effect cell-to-cell spread in an infected cell monolayer relative to wild-type *Listeria* can be determined by varying the concentration of gentamicin in the media containing agarose. Optionally, visualization and measurement of plaque diameter can be facilitated by the addition of media containing Neutral Red (GIBCO BRL; 1:250 dilution in DME+agarose media) to the overlay at 48 h. post infection. Additionally, the plaque assay can be performed in monolayers derived from other primary cells or continuous cells. For example HepG2 cells, a hepatocyte-derived cell line, or primary human hepatocytes can be used to evaluate the ability of selected *Listeria* mutants to effect cell-to-cell spread, as compared to wild-type *Listeria*. In some embodiments, *Listeria* comprising mutations or other modifications that attenuate the *Listeria* for cell-to-cell spread produce "pin-point" plaques at high concentrations of gentamicin (about 50 µg/ml).

[0279] In some embodiments, the bacteria comprising the expression cassettes and/or expression vectors described herein are mutant bacteria that are attenuated for nucleic acid repair (relative to wildtype such as bacteria without the attenuating genetic mutation). For instance, in some embodiments, the bacteria are defective with respect to at least one DNA repair enzyme (e.g., *Listeria monocytogenes* uvrAB mutants). In some embodiments, the bacteria are defective

with respect to PhrB, UvrA, UvrB, UvrC, UvrD, and/or RecA, or one of their equivalents (depending on the genus and species of the bacteria). In some embodiments, the bacteria are defective with respect to UvrA, UvrB, and/or UvrC. In some embodiments, the bacteria comprise attenuating mutations in phrB, uvrA, uvrB, uvrC, uvrD, and/or recA genes. In some embodiments, the bacteria comprise one or more mutations in the uvrA, uvrB, and/or uvrC genes. In some embodiments, the bacteria are functionally deleted in UvrA, UvrB, and/or UvrC. In some embodiments, the bacteria are deleted in functional UvrA and UvrB. In some embodiments, the bacteria are uvrAB deletion mutants. In some embodiments, the bacteria are Δ uvrAB Δ actA mutants. In some embodiments, the nucleic acid of the bacteria which are attenuated for nucleic acid repair and/or are defective with respect to at least one DNA repair enzyme are modified by reaction with a nucleic acid targeting compound (see below). Nucleic acid repair mutants, such as Δ uvrAB *Listeria monocytogenes* mutants, and methods of making the mutants, are described in detail in U.S. patent Publication No. 2004/0197343 (see, e.g., Example 7 of U.S. 2004/0197343).

[0280] In some embodiments, the capacity of the attenuated bacterium for nucleic acid repair is reduced by at least about 10%, at least about 25%, at least about 50%, at least about 75%, or at least about 90%, relative to a bacterium without the attenuating mutation (e.g., the wild type bacterium). In some embodiments, the capacity of the attenuated bacterium for nucleic acid repair is reduced by at least about 25% relative to a bacterium without the attenuating mutation. In some embodiments, the capacity of the attenuated bacterium for nucleic acid repair is reduced by at least about 50% relative to a bacterium without the attenuating mutation.

[0281] Confirmation that a particular mutation is present in a bacterial strain can be obtained through a variety of methods known to those of ordinary skill in the art. For instance, the relevant portion of the strain's genome can be cloned and sequenced. Alternatively, specific mutations can be identified via PCR using paired primers that code for regions adjacent to a deletion or other mutation. Southern blots can also be used to detect changes in the bacterial genome. Also, one can analyze whether a particular protein is expressed by the strain using techniques standard to the art such as Western blotting. Confirmation that the strain contains a mutation in the desired gene may also be obtained through comparison of the phenotype of the strain with a previously reported phenotype. For example, the presence of a nucleotide excision repair mutation such as deletion of uvrAB can be assessed using an assay which tests the ability of the bacteria to repair its nucleic acid using the nucleotide excision repair (NER) machinery and comparing that ability against wild-type bacteria. Such functional assays are known in the art. For instance, cyclobutane dimer excision or the excision of UV-induced (6-4) products can be measured to determine a deficiency in an NER enzyme in the mutant (see, e.g., Franklin et al., *Proc. Natl. Acad. Sci. USA*, 81: 3821-3824 (1984)). Alternatively, survival measurements can be made to assess a deficiency in nucleic acid repair. For instance, the bacteria can be subjected to psoralen/UVA treatment and then assessed for their ability to proliferate and/or survive in comparison to wild-type.

[0282] In some embodiments, the bacterium is attenuated for entry into non-phagocytic cells (relative to a non-mutant or wildtype bacterium). In some embodiments, the bacterium (e.g., *Listeria*) is defective with respect to one or more internalins (or equivalents). In some embodiments, the bacterium is defective with respect to internalin A. In some embodiments, the bacterium is defective with respect to internalin B. In some embodiments, the bacterium comprises a mutation in inlA. In some embodiments, the bacterium comprises a mutation in inlB. In some embodiments, the bacterium comprises a mutation in both actA and inlB. In some embodiments, the bacterium is deleted in functional ActA and internalinB. In some embodiments, the bacterium is an Δ actA Δ inlB double deletion mutant. In some embodiments, the bacterium is defective with respect to both ActA and internalin B.

[0283] In some embodiments, the capacity of the attenuated bacterium for entry into non-phagocytic cells is reduced by at least about 10%, at least about 25%, at least about 50%, at least about 75%, or at least about 90%, relative to a bacterium without the attenuating mutation (e.g., the wild type bacterium). In some embodiments, the capacity of the attenuated bacterium for entry into non-phagocytic cells is reduced by at least about 25% relative to a bacterium without the attenuating mutation. In some embodiments, the capacity of the attenuated bacterium for entry into non-phagocytic cells is reduced by at least about 50% relative to a bacterium without the attenuating mutation. In some embodiments, the capacity of the attenuated bacterium for entry into non-phagocytic cells is reduced by at least about 75% relative to a bacterium without the attenuating mutation.

[0284] In some embodiments, the attenuated bacteria, such as a mutant *Listeria* strain, are not attenuated for entry into more than one type of non-phagocytic cell. For instance, the attenuated strain may be attenuated for entry into hepatocytes, but not attenuated for entry into epithelial cells. As another example, the attenuated strain may be attenuated for entry into epithelial cells, but not hepatocytes. It is also understood that attenuation for entry into a non-phagocytic cell of particular modified bacteria is a result of mutating a designated gene, for example a deletion mutation, encoding an invasin protein which interacts with a particular cellular receptor, and as a result facilitates infection of a non-phagocytic cell. For example, *Listeria* Δ inlB mutant strains are attenuated for entry into non-phagocytic cells expressing the hepatocyte growth factor receptor (c-met), including hepatocyte cell lines (e.g., HepG2), and primary human hepatocytes.

[0285] In some embodiments, even though the bacteria (e.g., mutant *Listeria*) are attenuated for entry into non-phagocytic cells, the *Listeria* are still capable of uptake by phagocytic cells, such as at least dendritic cells and/or macrophages. In one embodiment the ability of the attenuated bacteria to enter phagocytic cells is not diminished by the modification made to the strain, such as the mutation of an invasin (i.e. approximately 95% or more of the measured ability of the strain to be taken up by phagocytic cells is maintained post-modification). In other embodiments, the ability of the attenuated bacteria to enter phagocytic cells is diminished by no more than about 10%, no more than about 25%, no more than about 50%, or no more than about 75%.

[0286] In some embodiments of the invention, the amount of attenuation in the ability of the bacterium (e.g., a *Listeria* bacterium) to enter non-phagocytic cells ranges from a two-fold reduction to much greater levels of attenuation. In some embodiments, the attenuation in the ability of the bacteria to enter non-phagocytic cells is at least about 0.3 log, about 1 log, about 2 log, about 3 log, about 4 log, about 5 log, or at least about 6 log. In some embodiments, the attenuation is in the range of about 0.3 to >8 log, about 2 to >8 log, about 4 to >8 log, about 6 to >8 log, about 0.3-8 log, also about 0.3-7 log, also about 0.3-6 log, also about 0.3-5 log, also about 0.3-4 log, also about 0.3-3 log, also about 0.3-2 log, also about 0.3-1 log. In some embodiments, the attenuation is in the range of about 1 to >8 log, 1-7 log, 1-6 log, also about 2-6 log, also about 2-5 log, also about 3-5 log.

[0287] A number of internalins have been identified in *L. monocytogenes* (Boland, et al., *Clinical Microbiology Reviews*, 2001, 14: 584-640). These internalins include, but are not limited to, InlA, InlB, InlC, InlC2, InlD, InlE, InlF, InlG, and InlH (Dramsi, et al., *Infection and Immunity*, 65: 1615-1625 (1997); Raffelsbauer et al., *Mol. Gen. Genet.* 260:144-158 (1988)). The gene sequences encoding these proteins have been previously reported. For instance, the sequences for both inlA and inlB have been reported in Gaillard et al., *Cell*, 65:1127-1141 (1991) and as GenBank accession number M67471. Genes encoding additional members of the internalin-related protein family are identified in Web Table 2 of the Supplementary Web material of Glaser et al., *Science*, 294:849-852 (2001), (www.sciencemag.org/cgi/content/full/294/5543/849/DC1), including lmo0327, lmo0331, lmo0514, lmo0610, lmo0732, lmo1136, lmo1289, lmo2396, lmo0171, lmo0333, lmo0801, lmo1290, lmo2026, and lmo2821. (The sequences of each member of the internalin-related protein family can be found in the *L. monocytogenes* strain EGD genome, GenBank Accession no. AL591824, and/or in the *L. monocytogenes* strain EGD-e genome, GenBank Accession no. NC_003210. Locations of the various internalin-related genes are indicated in Glaser et al.).

[0288] InlA (internalin A) (Gaillard et al., *Cell*, 65:1127-1141 (1991); Genbank accession no. NC_003210) directs the uptake of *Listeria* by epithelial cells such as those of the intestines.

[0289] InlB (internalin B) (Gaillard et al., *Cell*, 65:1127-1141 (1991); Genbank accession number AL591975 (*Listeria monocytogenes* strain EGD, complete genome, segment 3/12, inlB gene region: nts. 97008-98963); and Genbank accession number NC_003210 (*Listeria monocytogenes* strain EGD, complete genome, inlB gene region: nts. 457008-458963), each of which is incorporated by reference herein in its entirety) directs the uptake of *Listeria* by hepatocytes or by endothelial cells such as the vascular endothelial cells of the brain microvasculature that comprise the blood brain barrier. (For further descriptions of internalin B, see Ireton, et al., *J. of Biological Chemistry*, 274: 17025-17032 (1999); Dramsi, et al., *Molecular Microbiology* 16:251-261 (1995); Mansell et al., *J. of Biological Chemistry*, 276: 43597-43603 (2001); and Bierne et al., *J. of Cell Science* 115:3357-3367 (2002), all of which are incorporated by reference herein in their entirety.)

[0290] In some embodiments, the bacterium is deficient with respect to ActA, internalin B, or both Act A and

internalin B. In some embodiments, the bacterium is deleted in functional ActA, internalin B, or both ActA and internalin B. In some embodiments, the bacterium is deleted in functional ActA. In some embodiments, the bacterium is deleted in functional internalin B. In some embodiments, the bacterium is deleted in functional ActA and internalin B.

[0291] In vitro assays for determining whether or not a bacterium (e.g., a mutant *Listeria* strain) is attenuated for entry into non-phagocytic cells are known to those of ordinary skill in the art. For instance, both Dramsi et al., *Molecular Microbiology* 16:251-261 (1995) and Gaillard et al., *Cell* 65:1127-1141 (1991) describe assays for screening the ability of mutant *L. monocytogenes* strains to enter certain cell lines. For instance, to determine whether a *Listeria* bacterium with a particular modification is attenuated for entry into a particular type of non-phagocytic cells, the ability of the attenuated *Listeria* bacterium to enter a particular type of non-phagocytic cell is determined and compared to the ability of the identical *Listeria* bacterium without the modification to enter non-phagocytic cells. Likewise, to determine whether a *Listeria* strain with a particular mutation is attenuated for entry into a particular type of non-phagocytic cells, the ability of the mutant *Listeria* strain to enter a particular type of non-phagocytic cell is determined and compared to the ability of the *Listeria* strain without the mutation to enter non-phagocytic cells. In addition, confirmation that the strain is defective with respect to internalin B may also be obtained through comparison of the phenotype of the strain with the previously reported phenotypes for internalin B mutants.

[0292] In some embodiments, the attenuation of bacteria can be measured in terms of biological effects of the *Listeria* on a host. The pathogenicity of a bacterial strain can be assessed by measurement of the LD₅₀ in mice or other vertebrates. The LD₅₀ is the amount, or dosage, of *Listeria* injected into vertebrates necessary to cause death in 50% of the vertebrates. The LD₅₀ values can be compared for bacteria having a particular modification (e.g., mutation) versus the bacteria without the particular modification as a measure of the level of attenuation. For example, if the bacterial strain without a particular mutation has an LD₅₀ of 10³ bacteria and the bacterial strain having the particular mutation has an LD₅₀ of 10⁵ bacteria, the strain has been attenuated so that its LD₅₀ is increased 100-fold or by 2 log.

[0293] Alternatively, the degree of attenuation of the ability of a bacterium (e.g., a *Listeria* bacterium) to infect non-phagocytic cells can be assessed much more directly in vitro. For instance, the ability of a modified *Listeria* bacterium to infect non-phagocytic cells, such as hepatocytes, can be compared to the ability of non-modified *Listeria* or wild type *Listeria* to infect phagocytic cells. In such an assay, the modified and non-modified *Listeria* are typically added to the non-phagocytic cells in vitro for a limited period of time (for instance, an hour), the cells are then washed with a gentamicin-containing solution to kill any extracellular bacteria, the cells are lysed and then plated to assess titer. Examples of such an assay are found in U.S. patent publication No. 2004/0228877.

[0294] As a further example, the degree of attenuation may also be measured qualitatively by other biological effects, such as the extent of tissue pathology or serum liver enzyme levels. Alanine aminotransferase (ALT), aspartate

aminotransferase (AST), albumin and bilirubin levels in the serum are determined at a clinical laboratory for mice injected with *Listeria* (or other bacteria). Comparisons of these effects in mice or other vertebrates can be made for *Listeria* with and without particular modifications/mutations as a way to assess the attenuation of the *Listeria*. Attenuation of the *Listeria* may also be measured by tissue pathology. The amount of *Listeria* that can be recovered from various tissues of an infected vertebrate, such as the liver, spleen and nervous system, can also be used as a measure of the level of attenuation by comparing these values in vertebrates injected with mutant versus non-mutant *Listeria*. For instance, the amount of *Listeria* that can be recovered from infected tissues such as liver or spleen as a function of time can be used as a measure of attenuation by comparing these values in mice injected with mutant vs. non-mutant *Listeria*.

[0295] Accordingly, the attenuation of the *Listeria* can be measured in terms of bacterial load in particular selected organs in mice known to be targets by wild-type *Listeria*. For example, the attenuation of the *Listeria* can be measured by enumerating the colonies (Colony Forming Units; CFU) arising from plating dilutions of liver or spleen homogenates (homogenized in H₂O+0.2% NP40) on BHI agar media. The liver or spleen cfu can be measured, for example, over a time course following administration of the modified *Listeria* via any number of routes, including intravenous, intraperitoneal, intramuscular, and subcutaneous. Additionally, the *Listeria* can be measured and compared to a drug-resistant, wild type *Listeria* (or any other selected *Listeria* strain) in the liver and spleen (or any other selected organ) over a time course following administration by the competitive index assay, as described.

[0296] The degree of attenuation in uptake of the attenuated bacteria by non-phagocytic cells need not be an absolute attenuation in order to provide a safe and effective vaccine. In some embodiments, the degree of attenuation is one that provides for a reduction in toxicity sufficient to prevent or reduce the symptoms of toxicity to levels that are not life threatening.

[0297] In some embodiments of the invention, the bacterium that comprises a recombinant nucleic acid molecule, expression cassette and/or expression vector described herein is a mutant strain of *Listeria*. In further embodiments, the bacterium is an attenuated mutant strain of *Listeria monocytogenes*. A variety of exemplary mutant strains of *Listeria* that are attenuated are described in the U.S. Patent Application Nos. 60/446,051 (filed Feb. 6, 2003), 60/449,153 (filed Feb. 21, 2003), 60/511,719 (filed Oct. 15, 2003), 60/511,919 (filed Oct. 15, 2003), 60/511,869 (filed Oct. 15, 2003), 60/541,515 (filed Feb. 2, 2004), and Ser. No. 10/883,599 (filed Jun. 30, 2004), as well as in U.S. patent Publication Nos. 2004/0197343 and US 2004/0228877, each of which is incorporated by reference herein in its entirety. Mutant strains of *Listeria* are also described in the International Application No. PCT/US2004/23881, filed Jul. 23, 2004, which is incorporated by reference herein in its entirety. For instance, the bacterium that comprise the expression cassette and/or vector is optionally a mutant strain of *Listeria monocytogenes* that is defective with respect to ActA or internalin B, or both ActA and internalin B. In some embodiments, the bacterium is a mutant strain of *Listeria monocytogenes* that is actA⁻ (e.g., DP-L4029 (the DP-L3078 strain described in Skoble et al., *J. of Cell*

Biology, 150: 527-537 (2000), incorporated by reference herein in its entirety, which has been cured of its prophage as described in (Lauer et al., *J. Bacteriol.* 184:4177 (2002); U.S. patent Publication No. 2003/0203472)), actA⁻inlB⁻ (e.g., DP-L4029inlB, deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Va. 20110-2209, United States of America, on Oct. 3, 2003, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and designated with accession number PTA-5562), actA⁻uvrAB⁻ (e.g., DP-L4029uvrAB, deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Va. 20110-2209, United States of America, on Oct. 3, 2003, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and designated with accession number PTA-5563), or actA⁻inlB⁻uvrAB⁻. In some embodiments, the attenuated *Listeria* bacterium (e.g., a *Listeria monocytogenes* bacterium) is an Δ actA Δ inlB double deletion mutant.

[0298] Bacterial mutations can be achieved through traditional mutagenic methods, such as mutagenic chemicals or radiation followed by selection of mutants. Bacterial mutations can also be achieved by one of skill in the art through recombinant DNA technology. For instance, the method of allelic exchange using the pKSV7 vector described in Camilli et al., *Molecular Micro.* 8:143-157 (1993) and described above with respect to the introduction of heterologous expression cassettes in bacterial genomes is suitable for use in generating mutants including deletion mutants. (Camilli et al. (1993) is incorporated by reference herein in its entirety.) One exemplary example of the production of a *Listeria monocytogenes* internalin B mutant using the pKSV7 vector is provided in Example 24, below. Alternatively, the gene replacement protocol described in Biswas et al., *J. Bacteriol.* 175:3628-3635 (1993), can be used. Other similar methods are known to those of ordinary skill in the art.

[0299] The construction of a variety of bacterial mutants is described in U.S. patent application Ser. No. 10/883,599, U.S. patent Publication No. 2004/0197343, and U.S. patent Publication No. 2004/0228877, each of which is incorporated by reference herein in its entirety.

[0300] In some embodiments of the invention, the bacterium that comprises the recombinant nucleic acid molecule, expression cassette and/or expression vector is a mutant strain of *Bacillus anthracis*. In some embodiments, the bacterium is the Sterne strain. In some embodiments, the bacterium is the Ames strain. In some embodiments, the *Bacillus anthracis* bacterium is a uvrAB mutant. In some embodiments, the *Bacillus anthracis* strain is a uvrC mutant. In some embodiments, the *Bacillus anthracis* mutant is a recA mutant. In some embodiments, the bacterium is a JuvrAB mutant of the *Bacillus anthracis* (e.g., the *Bacillus anthracis* Sterne Δ uvrAB mutant deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Va. 20110-2209, United States of America, on Feb. 20, 2004, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and designated with accession number PTA-5825).

[0301] Methods of altering the genome of *Bacillus anthracis* are known to those skilled in the art. One method of generating mutations in *Bacillus anthracis* is by allelic exchange using an allelic exchange vector known to those in the art. An exemplary allelic exchange plasmid is pKSV7 described in Camilli et al., *Molecular Microbiology*, 8:143-147 (1993). As a first step in generating a mutant *Bacillus anthracis*, the region of the genome to be deleted or otherwise mutated and approximately 1000 bps both upstream and downstream of the *B. anthracis* genome is PCR-amplified and then cloned into the pKSV7 plasmid vector (or an analogous vector). (A *Bacillus* genera-specific or *B. anthracis*-specific temperature (ts) replicon may be substituted for the *Listeria* ts replicon present in the pKSV7 allelic exchange plasmid vector.) Restriction endonuclease recognition sites in the region to be deleted or mutated may be used to delete the desired portion of the targeted gene in the region. Alternatively, a portion of the targeted gene within the region may be removed and replaced with sequences containing the desired mutation or other alteration. The region of the *B. anthracis* genome that is amplified can be altered, for instance, using restriction enzymes or a combination of restriction enzymes and synthetic gene sequences, before or after cloning into the allelic exchange plasmid. In some embodiments, the sequence may be altered as a PCR amplicon and then cloned into pKSV7. In alternative embodiments, the amplicon is first inserted into another plasmid first and then altered, excised, and inserted into pKSV7. Alternatively, the PCR amplicon is inserted directly into the pKSV7 plasmid and then altered, for instance, using convenient restriction enzymes. The pKSV7 plasmid containing the altered sequence is then introduced into *B. anthracis*. This can be done via electroporation. The bacteria are then selected on media at a permissive temperature in the presence of chloramphenicol. This is followed by selection for single cross-over integration into the bacterial chromosome by passaging for multiple generations at a non-permissive temperature in the presence of chloramphenicol. Lastly, colonies are passaged for multiple generations at the permissive temperature in media not containing the antibiotic to achieve plasmid excision and curing (double cross-over). The U.S. Provisional Application Ser. Nos. 60/584, 886, and 60/599,522, and U.S. patent Publication No. 2004/0197343, incorporated by reference herein in their entirety, provide additional description regarding the construction of different types of *Bacillus anthracis* mutants.

[0302] In some embodiments of the invention, the bacterium that comprises the recombinant nucleic acid molecule, expression cassette, and/or expression vector is a bacterium that has been modified so that the bacterium is attenuated for proliferation (relative to the non-modified bacterium). Preferably, the modified bacterium maintains a sufficient level of gene expression despite the modification. For instance, in some embodiments the gene expression level is substantially unaffected by the modification so that an antigen is expressed at a level sufficient to stimulate an immune response to the antigen upon administration of the bacterium expressing the antigen to a host. In some embodiments, the nucleic acid of the bacterium has been modified by reaction with a nucleic acid targeting compound. In some embodiments, the nucleic acid of the modified bacterium has been modified by reaction with a nucleic acid targeting compound that reacts directly with the nucleic acid so that proliferation of the bacterium is attenuated. In some embodiments, the

nucleic-acid targeting compound is a nucleic acid alkylator, such as β -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl-ester. In some embodiments, the nucleic acid targeting compound is activated by irradiation, such as UVA irradiation. In some embodiments, the bacterium has been treated with a psoralen compound. For instance, in some embodiments, the bacterium has been modified by treatment with a psoralen, such as 4'-(4-amino-2-oxa)butyl-4,5',8'-trimethylpsoralen ("S-59"), and UVA light. In some embodiments, the nucleic acid of the bacterium has been modified by treatment with a psoralen compound and UVA irradiation. Descriptions of methods of modifying bacteria to attenuate them for proliferation using nucleic acid targeting compounds are described in each of the following U.S. patent applications or publications, each of which is incorporated by reference herein in its entirety: 60/446,051 (filed Feb. 6, 2003), 60/449,153 (filed Feb. 21, 2003), 60/490,089 (filed Jul. 24, 2003), 60/511,869 (filed Oct. 15, 2003), 60/541,515 (filed Feb. 2, 2004), 10/883,599 (filed Jun. 30, 2004), and US 2004/0197343. Modified bacteria and their uses are also described in International Application No. PCT/US2004/23881, filed Jul. 23, 2004, incorporated by reference herein in its entirety.

[0303] For example, for treatment of $\Delta actA \Delta uvrAB$ *L. monocytogenes*, in some embodiments, S-59 psoralen can be added to 200 nM in a log-phase culture of (approximately) $OD_{600}=0.5$, followed by inactivation with 6 J/m² of UVA light when the culture reaches an optical density of one. Inactivation conditions are optimized by varying concentrations of S-59, UVA dose, the time of S-59 exposure prior to UVA treatment as well as varying the time of treatment during bacterial growth of the *Listeria actA/uvrAB* strain. The parental *Listeria* strain is used as a control. Inactivation of *Listeria* (log-kill) is determined by the inability of the bacteria to form colonies on BHI (Brain heart infusion) agar plates. In addition, one can confirm the expression of a heterologous protein and virulence factors, such as LLO and p60, of the S-59/UVA inactivated *Listeria* using ³⁵S-pulse-chase experiments to determine the synthesis and secretion of newly expressed proteins post S-59/UVA inactivation. Expression of LLO and p60 using ³⁵S-metabolic labeling can be routinely determined. S-59/UVA inactivated *Listeria actA/uvrAB* can be incubated for 1 hour in the presence of ³⁵S-Methionine. Antigen expression and secretion of the heterologous protein, endogenous LLO, and p60 can be determined of both whole cell lysates, and TCA precipitation of bacterial culture fluids. LLO-, p60- and heterologous protein-specific monoclonal antibodies can be used for immunoprecipitation to verify the continued expression and secretion from recombinant *Listeria* post inactivation.

[0304] In some embodiments, the bacteria attenuated for proliferation are also attenuated for nucleic acid repair and/or are defective with respect to at least one DNA repair enzyme. For instance, in some embodiments, the bacterium in which nucleic acid has been modified by a nucleic acid targeting compound such as a psoralen (combined with UVA treatment) is a *uvrAB* deletion mutant.

[0305] In some embodiments, the proliferation of the bacteria is attenuated by at least about 0.3 log, also at least about 1 log, about 2 log, about 3 log, about 4 log, about 6 log, or at least about 8 log. In another embodiment, the proliferation of the bacteria is attenuated by about 0.3 to >10 log, about 2 to >10 log, about 4 to >10 log, about 6 to >10

log, about 0.3-8 log, about 0.3-6 log, about 0.3-5 log, about 1-5 log, or about 2-5 log. In some embodiments, the expression of an antigen by the bacteria are at least about 10%, about 25%, about 50%, about 75%, or at least about 90% of the expression of the antigen by bacteria in which the bacterial nucleic acid is not modified.

[0306] In some embodiments, the nucleic acid of the bacterium has not been modified by reaction with a nucleic acid targeting compound. In some embodiments, the recombinant bacterium has not been attenuated for proliferation. In some embodiments, the recombinant bacterium is not attenuated in its ability for nucleic acid repair. In some embodiments, the recombinant bacterium is not deficient with respect to at least one DNA repair enzyme.

[0307] In some embodiments, the signal peptide encoded by first polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or expression vector contained within the recombinant bacterium is native to the recombinant bacterium. In some embodiments, the polynucleotide encoding the signal peptide that is native to the recombinant bacterium has been codon-optimized for expression in the recombinant bacterium. In some embodiments, the polynucleotide has been fully codon-optimized. In some embodiments, the signal peptide encoded by the first polynucleotide of the recombinant nucleic acid molecule, expression cassette, and/or expression vector contained within the recombinant bacterium is foreign to the host recombinant bacterium. In some embodiments, the polynucleotide encoding the signal peptide that is foreign to the host recombinant bacterium has been codon-optimized for expression in the recombinant bacterium.

[0308] In some embodiments, the second polynucleotide in the recombinant nucleic acid molecule, expression cassette, and/or expression vector within the recombinant bacterium has been codon-optimized for expression in the recombinant bacterium. In some embodiments, the second polynucleotide has been fully codon-optimized for expression in the recombinant bacterium. In some embodiments, both the first and second polynucleotides within the recombinant bacterium have been codon-optimized for expression in the recombinant bacterium. In some embodiments, both the first and second polynucleotides within the recombinant bacterium have been fully codon-optimized for expression in the recombinant bacterium.

[0309] In one aspect, the invention provides a bacterium comprising an expression cassette, wherein the expression cassette comprises (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in the bacterium; (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising the signal peptide and the polypeptide. As described herein, e.g., in Section III, in some embodiments, the signal peptide that is encoded is a derived from bacteria. In some embodiments, the bacterial signal peptide encoded by the expression cassette is derived from the bacteria of the same genus and/or species as the bacterium comprising the expression cassette. In some embodiments, the signal peptide is native to the host recombinant bacterium. In other embodiments,

the bacterial signal peptide encoded by the expression cassette is derived from bacteria of a different genus and/or species as the bacterium comprising the expression cassette. In some embodiments, the signal peptide is foreign to the host recombinant bacterium. In some embodiments the signal peptide is a secA1, secA2, or a Tat signal peptide. In some embodiments the polypeptide encoded by the second polynucleotide is heterologous (i.e., foreign) to the bacterium.

[0310] In another aspect, the invention provides a bacterium comprising a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide native to the bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the bacterium is an intracellular bacterium. In some embodiments, the recombinant nucleic acid molecule is part of an expression cassette that further comprises a promoter operably linked to both the first and second polynucleotides. In some embodiments, the bacterium is selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria and *E. coli*. In some embodiments, the bacterium is *Listeria* (e.g., *Listeria monocytogenes*).

[0311] In another aspect, the invention provides a recombinant *Listeria* bacterium (e.g., *Listeria monocytogenes*) comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in the *Listeria* bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the recombinant nucleic acid molecule is part of an expression cassette that further comprises a promoter operably linked to both the first and second polynucleotides. In some embodiments, the second polynucleotide is codon-optimized for expression in the *Listeria* bacterium. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the *Listeria* bacterium (i.e., heterologous to the *Listeria* bacterium). In some embodiments, the *Listeria* bacterium is attenuated. For instance, the *Listeria* may be attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the recombinant *Listeria* bacterium is deficient with respect to ActA, Internalin B, or both Act A and Internalin B (e.g., an Δ actA Δ inlB double deletion mutant). In some embodiments, the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound (e.g., a psoralen compound).

[0312] In another aspect, the invention provides a bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a first polynucleotide encoding a non-secA1 bacterial signal peptide, and a second polynucleotide encoding a polypeptide (e.g., an antigen), wherein the second polynucleotide is

in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide. In some embodiments, the recombinant nucleic acid molecule is part of an expression cassette that further comprises a promoter operably linked to both the first and second polynucleotides. In some embodiments, the bacterium is a bacterium selected from the group consisting of *Listeria*, *Bacillus*, *Yersinia pestis*, *Salmonella*, *Shigella*, *Brucella*, mycobacteria or *E. coli*. In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the bacterium (i.e., heterologous to the bacterium).

[0313] In another aspect, the invention provides a bacterium comprising an expression cassette, wherein the expression cassette comprises (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; (b) a second polynucleotide encoding a polypeptide (e.g., a polypeptide heterologous to the bacterium) in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising the signal peptide and the polypeptide. As described herein, e.g., in Section III, above, in some embodiments, the non-secA1 bacterial signal peptide is a secA2 signal peptide. In some other embodiments, the non-secA1 bacterial signal peptide is a Tat signal peptide. In some embodiments, the bacterial signal peptide encoded by the expression cassette is derived from the bacteria of the same genus and/or species as the bacterium comprising the expression cassette. In other embodiments, the bacterial signal peptide encoded by the expression cassette is derived from bacteria of a different genus and/or species as the bacterium comprising the expression cassette.

[0314] In another aspect, the invention provides a recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the recombinant nucleic acid molecule is part of an expression cassette that further comprises a promoter operably linked to both the first and second polynucleotides. In some embodiments, the *Listeria* bacterium is attenuated. In some embodiments, the *Listeria* bacterium is a *Listeria monocytogenes* bacterium. For instance, the *Listeria* may be attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the recombinant *Listeria* bacterium is deficient with respect to ActA, Internalin B, or both Act A and Internalin B (e.g., an Δ actA Δ inlB double deletion mutant). In some embodiments, the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound (e.g., a psoralen compound).

[0315] In another aspect, the invention provides a recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a polynucleotide encoding a polypep-

tide foreign to the *Listeria* bacterium, wherein the polynucleotide is codon-optimized for expression in *Listeria*.

[0316] In an additional aspect, the invention provides a recombinant *Listeria* bacterium comprising an expression cassette, wherein the expression cassette comprises the following: (a) a polynucleotide encoding a polypeptide foreign to the *Listeria* bacterium, wherein the polynucleotide is codon-optimized for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the foreign polypeptide. Again, in some embodiments, the *Listeria* is *Listeria monocytogenes*. In other embodiments the *Listeria* bacterium belongs to the *Listeria ivanovii*, *Listeria seeligeri*, or *Listeria innocua* species. In some embodiments, the *Listeria* bacterium is an attenuated strain of *Listeria* bacterium as described above.

[0317] In a further aspect, the invention provides a recombinant *Listeria* bacterium (e.g., *Listeria monocytogenes*) comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-*Listeria* signal peptide; and (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-*Listeria* signal peptide and the polypeptide. In some embodiments, the *Listeria* bacterium is attenuated. For instance, the *Listeria* may be attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the recombinant *Listeria* bacterium is deficient with respect to ActA, Internalin B, or both Act A and Internalin B (e.g., an Δ actA Δ inlB double deletion mutant). In some embodiments, the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound (e.g., a psoralen compound).

[0318] In still another aspect, the invention provides a recombinant *Listeria* bacterium (for instance, from the species *Listeria monocytogenes*) comprising an expression cassette which comprises a first polynucleotide encoding a non-*Listeria* signal peptide, a second polynucleotide encoding a polypeptide (e.g., a non-*Listeria* polypeptide) that is in the same translational reading frame as the first polynucleotide, and a promoter operably linked to both the first and second polynucleotides. The expression cassette encodes a fusion protein comprising both the non-*Listeria* signal peptide and the polypeptide. In some embodiments, the *Listeria* bacterium is attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation. In some embodiments, the *Listeria* bacterium is deficient with respect to ActA, Internalin B, or both ActA and Internalin B. In some embodiments, the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound (e.g., a psoralen compound). In some embodiments, the first polynucleotide, the second polynucleotide, or both the first and second polynucleotides are codon-optimized for expression in *Listeria*. In some embodiments, the first polynucleotide and/or second polynucleotide is codon-optimized for expression in *Listeria monocytogenes*. In some embodiments, the polypeptide encoded by the second polynucleotide is an antigen, which, in some instances, may be a non-bacterial antigen. For instance, the polypeptide is, in some embodiments a tumor-associated antigen or is derived from such a tumor-associated antigen. For instance, in some embodiments, the polypeptide is

K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA, or is derived from K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, or CEA. For instance, in some embodiments, the polypeptide is mesothelin, or is a fragment or variant of mesothelin. In some other embodiments, the polypeptide is NY-ESO-1, or a fragment or variant of mesothelin. In some other embodiments, the antigen is an infectious disease antigen or is derived from an infectious disease antigen. In preferred embodiments, the signal peptide is bacterial. In some embodiments, the signal peptide is from a bacterium belonging to the genus *Bacillus*, *Staphylococcus*, or *Lactococcus*. For instance, in some embodiments, the signal peptide is from *Bacillus anthracis*, *Bacillus subtilis*, *Staphylococcus aureus*, or *Lactococcus lactis*. In some embodiments, the signal peptide is a secA1 signal peptide, such as a Usp45 signal peptide from *Lactococcus lactis* or a Protective Antigen signal peptide from *Bacillus anthracis*. In some embodiments, the signal peptide is a secA2 signal peptide. In still further embodiments, the signal peptide is a Tat signal peptide, such as a *B. subtilis* Tat signal peptide (e.g., PhoD).

[0319] The invention further provides a recombinant bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises: (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof; and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the polypeptide encoded by the second polynucleotide and the autolysin, or catalytically active fragment or catalytically active variant thereof, wherein, in the protein chimera, the polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof, or is positioned within the autolysin, or catalytically active fragment or catalytically active variant thereof. In some embodiments, the recombinant bacterium is an intracellular bacterium, such as a *Listeria* bacterium (e.g., *Listeria monocytogenes*). In some embodiments, the polypeptide encoded by the second polynucleotide is foreign to the recombinant bacterium.

[0320] In yet another aspect, the invention provides a recombinant *Listeria* bacterium comprising a polycistronic expression cassette, wherein the polycistronic expression cassette encodes at least two discrete non-*Listeria* polypeptides. For instance, in some embodiments, the expression cassette comprises a first polynucleotide encoding the first non-*Listeria* polypeptide, a second polynucleotide encoding the second non-*Listeria* polypeptide, and a promoter operably linked to the first and second polynucleotides. In some embodiments, the recombinant *Listeria* bacterium belongs to the species *Listeria monocytogenes*. In some embodiments, the first and/or second non-*Listeria* polypeptides comprise antigens (or fragments thereof).

[0321] In some embodiments, the invention provides a recombinant bacterium (e.g., *Listeria*) comprising an expression cassette comprising the following: (a) a first polynucleotide encoding a first signal peptide; (b) a second polynucleotide encoding a first polypeptide, wherein the

second polynucleotide is in the same translational reading frame as the first polynucleotide; (c) an intergenic sequence; (d) a third polynucleotide encoding a second signal peptide; (e) a fourth polynucleotide encoding a second polypeptide, wherein the fourth polynucleotide is in the same translational reading frame as the third polynucleotide; and (f) a promoter operably linked to the first polynucleotide, second polynucleotide, third polynucleotide, fourth polynucleotide, and intergenic sequence, such that the expression cassette encodes both a first fusion protein comprising the first signal peptide and the first polypeptide and a second fusion protein comprising the second signal peptide and second polypeptide. In some embodiments, the one or more of the polynucleotides encoding a signal peptide is codon-optimized for expression in the bacterium. In some embodiments, the third and/or fourth polynucleotides are codon-optimized for expression in the bacterium. In some embodiments, the first and/or second polypeptides are heterologous to the recombinant bacterium (e.g., heterologous antigens). In some embodiments, the first and/or second signal peptide is a non-secA1 bacterial signal peptide. The first and/or second signal peptide may be native or foreign to the recombinant bacterium. In some embodiments, the recombinant bacterium is a *Listeria* bacterium and the first and/or second signal peptide is non-*Listeria*. In some embodiments, the intergenic sequence is the *Listeria monocytogenes* actA-plcB intergenic sequence. In some embodiments, the bacterium is *Listeria monocytogenes*.

[0322] In other aspects, the invention provides a bacterium comprising a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a signal peptide, (b) a second polynucleotide encoding a secreted protein, or a fragment thereof, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a third polynucleotide encoding a polypeptide heterologous to the secreted protein, or fragment thereof, wherein the third polynucleotide is in the same translational reading frame as the first and second polynucleotides, and wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the second polynucleotide, and the secreted protein, or fragment thereof, and wherein the polypeptide is fused to the secreted protein, or fragment thereof, or is positioned within the secreted protein, or fragment thereof, in the protein chimera. In some embodiments, the bacterium is a *Listeria* bacterium. In some embodiments, where the bacterium is a *Listeria* bacterium, the polypeptide encoded by the third polynucleotide is foreign to the *Listeria*. In some embodiments, the bacterium is *Listeria monocytogenes*.

[0323] In some embodiments (for instance, in some embodiments of each of the aforementioned aspects), the expression cassette contained within the bacterium is integrated into the genome of the bacterium. In other embodiments, the expression cassette contained within the bacterium is on a plasmid within the bacterium.

[0324] Generally, the promoter that is used in the expression cassette will be an expression cassette that is suitable for effecting heterologous expression with the particular bacterial host chosen. One of ordinary skill in the art can readily discern which promoters are suitable for use in which bacteria. In some embodiments, the promoter is a bacterial promoter. In additional embodiments, the promoter on the expression cassette in the bacterium is a promoter

from bacteria belonging to the same genus as the bacterium which contains the expression cassette. In other embodiments, the promoter on the expression cassette in the bacterium is a promoter from bacteria belonging to the same species as the bacterium which contains the expression cassette. For instance, if the bacterium which contains the expression cassette belongs to the species *Listeria monocytogenes*, then the promoter that is used on the expression cassette is optionally derived from a Listerial gene such as hly. In other embodiments, the promoter is a constitutive promoter (e.g., a p60 promoter) or is prfA-dependent promoter (e.g. an actA promoter). Again, as described above, the promoter of the expression cassette is, in some embodiments, a constitutive promoter. In other embodiments, the promoter of the expression cassette is an inducible promoter, as also described above.

[0325] In some embodiments (for instance, in some embodiments of each of the aforementioned aspects), the polypeptides or fusion proteins comprising the polypeptides that are encoded by the expression cassettes in the bacteria are antigens or other proteins of therapeutic value, as described, for instance, above in Section IV. In some embodiments, the polypeptide or a protein comprising the polypeptide is secreted from the bacterium. In some embodiments the polypeptide that is expressed and/or secreted from the bacterium is heterologous to the bacterium.

[0326] In some embodiments, therefore, the invention provides recombinant *Listeria* comprising an expression cassette, wherein the expression cassette comprises (a) a first polynucleotide encoding a bacterial (either Listerial or non-Listerial) signal peptide, wherein the first polynucleotide is codon-optimized for expression in *Listeria*; (b) a second polynucleotide encoding a non-Listerial antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising the signal peptide and the antigen. In further embodiments, the *Listeria* is a strain of *Listeria monocytogenes*, such as an actA⁻inlB⁻ strain. In some embodiments, the expression cassette has been integrated into the genome of the recombinant *Listeria*. In some embodiments, the second polynucleotide is codon-optimized for expression in *Listeria*.

[0327] The invention also provides *Listeria* comprising an expression cassette, wherein the expression cassette comprises (a) a first polynucleotide encoding a secA2 or Tat bacterial signal peptide; (b) a second polynucleotide encoding an antigen in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising the signal peptide and the antigen. In some embodiments, the bacterial signal peptide is Listerial. In other embodiments, the bacterial signal peptide is non-Listerial. In further embodiments, the *Listeria* is a strain of *Listeria monocytogenes*, such as an actA⁻inlB⁻ strain. In some embodiments, the expression cassette has been integrated into the genome of the recombinant *Listeria*. In some embodiments, either the polynucleotide encoding the signal peptide (even if the signal peptide is a Listerial signal peptide) and/or the polynucleotide encoding the antigen is codon-optimized for expression in *Listeria*.

[0328] In further embodiments, the invention provides recombinant *Listeria* comprising an expression cassette, wherein the expression cassette comprises the following: (a) a polynucleotide encoding a non-Listerial antigen, wherein the polynucleotide is codon-optimized for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the foreign polypeptide. In some embodiments, the expression cassette further comprises a polynucleotide encoding a bacterial signal peptide, which is also codon-optimized for expression in *Listeria*. In one embodiment, the bacterial signal peptide is Listerial. In another embodiment, the bacterial signal peptide is non-Listerial. In some embodiments the bacterial signal peptide is a secA1 signal peptide, a secA2 signal peptide, or a Tat signal peptide. In further embodiments, the *Listeria* is a strain of *Listeria monocytogenes*, such as an actA⁻inlB⁻ strain. In some embodiments, the expression cassette has been integrated into the genome of the recombinant *Listeria*.

[0329] In still another embodiment, the invention provides a recombinant *Listeria* bacterium, comprising (a) a first polynucleotide encoding a bacterial (either Listerial or non-Listerial) signal peptide, wherein the first polynucleotide is codon-optimized for expression in *Listeria*; (b) a second polynucleotide encoding an non-Listerial antigen, wherein the second polynucleotide is also codon-optimized for expression in *Listeria* and is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising the signal peptide and the antigen. In some embodiments, the *Listeria* bacterium belongs to the species *Listeria monocytogenes*. In some embodiments, the *Listeria* bacterium is an actA⁻inlB⁻ mutant strain of *Listeria monocytogenes*.

[0330] The present invention further provides bacteria such as *Listeria* comprising more than one expression cassette described herein. In particular compositions, the molecular mass of a given protein can inhibit its expression from recombinant bacteria, such as recombinant *Listeria*. One approach to address this problem is to molecularly "divide" the gene encoding a protein of interest and fuse each division functionally to a sequence that will program its secretion from the bacterium (e.g., secA1, secA2, or Tat elements). One approach is to individually derive recombinant *Listeria* expressing each division of the heterologous gene. Alternatively, the individually components of the molecularly divided gene (also including appropriate elements for secretion) can be introduced into intergenic regions throughout the bacterial chromosome, using methods well established in the art, for example by allelic exchange. Another example is to express the molecularly divided gene as a single polycistronic message. According to this composition, interspersed between the protein-encoding sequence of the molecularly divided gene would be the Shine-Dalgarno ribosome binding sequence, in order to re-initiate protein synthesis on the polycistronic message.

[0331] In additional aspects, the invention provides methods of improving expression and/or secretion of heterologous polypeptides in recombinant bacteria such as *Listeria*. Any of the polynucleotides, expression cassettes and/or expression vectors described herein may be used in these methods. For instance, the invention provides a method of improving expression and/or secretion of a heterologous

polypeptide fused to a signal peptide in *Listeria*, comprising codon-optimizing either the polypeptide-encoding sequence on the expression cassette, the signal peptide-encoding sequence of the expression cassette, or both. The invention also provides a method of improving expression and/or secretion of a heterologous polypeptide fused to a signal peptide in *Listeria*, comprising using a signal peptide from a non-*Listerial* source and/or from a secretory pathway other than secA1.

[0332] The invention also provides a method of producing a recombinant bacterium (e.g. a recombinant *Listeria* bacterium) comprising introducing a recombinant nucleic acid molecule, expression cassette, and/or expression vector described herein into a bacterium to produce the recombinant bacterium. For instance, in some embodiments, a recombinant nucleic acid molecule comprising (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide, is introduced into a bacterium to produce the recombinant bacterium. In some embodiments, a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide, is introduced into a bacterium to produce the recombinant bacterium. In some embodiments, the recombinant nucleic acid molecule that is introduced into a bacterium to produce the recombinant bacterium is a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-*Listerial* signal peptide, and (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-*Listerial* signal peptide and the polypeptide. The recombinant nucleic acid molecule used to produce the recombinant bacterium is, in some embodiments, a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a bacterial autolysin, or catalytically active fragment or catalytically active variant thereof, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a protein chimera in which the non-*Listerial* polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof, or is inserted within the autolysin, or catalytically active fragment or catalytically active variant thereof. In some other embodiments, a method of producing a recombinant *Listeria* bacterium is provided, which comprises introducing a polycistronic expression cassette, wherein the expression cassette encodes at least two discrete non-*Listerial* polypeptides, into a *Listeria* bacterium to produce the recombinant *Listeria* bacterium.

[0333] IX. Pharmaceutical, Immunogenic, and/or Vaccine Compositions

[0334] A variety of different compositions such as pharmaceutical compositions, immunogenic compositions, and vaccines are also provided by the invention. These compositions comprise any of the recombinant bacteria described herein (see, e.g., the Summary of the Invention, Sections I and VIII of the Detailed Description, above, and elsewhere in the specification, including the Examples, below). In some embodiments, the compositions are isolated.

[0335] For instance, the invention provides a pharmaceutical composition comprising the following: (i) a pharmaceutically acceptable carrier; and (ii) a recombinant bacterium described herein.

[0336] For example, the invention provides a pharmaceutical composition comprising the following (i) a pharmaceutically acceptable carrier; and (ii) a recombinant bacterium comprising an expression cassette comprising a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium, a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the polypeptide.

[0337] The invention also provides a pharmaceutical composition comprising: (i) a pharmaceutically acceptable carrier; and (ii) a recombinant bacterium comprising an expression cassette, where the expression cassette comprises a first polynucleotide encoding a non-secA1 bacterial signal peptide, a second polynucleotide encoding a polypeptide in the same translational reading frame as the first polynucleotide, and a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the polypeptide.

[0338] The invention further provides a pharmaceutical composition comprising: (i) a pharmaceutically acceptable carrier; and (ii) a recombinant *Listeria* bacterium comprising an expression cassette, wherein the expression cassette comprises the following: (a) a polynucleotide encoding a polypeptide foreign to *Listeria*, wherein the polynucleotide is codon-optimized for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the foreign polypeptide.

[0339] The invention also provides a pharmaceutical composition comprising: (i) a pharmaceutically acceptable carrier; and (ii) a recombinant *Listeria* bacterium comprising an expression cassette which comprises: (a) a first polynucleotide encoding a non-*Listerial* signal peptide; (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to both the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising both the non-*Listerial* signal peptide and the polypeptide.

[0340] As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, anti-fungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. Pharmaceutically acceptable carriers are well known to those of ordinary skill in the art, and include any material

which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. For instance, pharmaceutically acceptable carriers include, but are not limited to, water, buffered saline solutions (e.g., 0.9% saline), emulsions such as oil/water emulsions, and various types of wetting agents. Possible carriers also include, but are not limited to, oils (e.g., mineral oil), dextrose solutions, glycerol solutions, chalk, starch, salts, glycerol, and gelatin.

[0341] While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions, the type of carrier will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. In some embodiments, for parenteral administration, such as subcutaneous injection, the carrier comprises water, saline, alcohol, a fat, a wax or a buffer. In some embodiments, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, are employed for oral administration.

[0342] Compositions comprising such carriers are formulated by well known conventional methods (see, for example, *Remington's Pharmaceutical Sciences*, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990; and *Remington, The Science and Practice of Pharmacy* 20th Ed. Mack Publishing, 2000).

[0343] In addition to pharmaceutical compositions, immunogenic compositions are provided. For instance, the invention provides an immunogenic composition comprising a recombinant bacterium described herein (see, e.g., the recombinant bacteria described above in the Summary of the Invention, Sections I and VIII of the Detailed Description above, and elsewhere in the specification, including the Examples, below). In some embodiments, the immunogenic composition comprises a recombinant bacterium, wherein the polypeptide sequence that is part of the polypeptide expressed by the recombinant bacterium as a discrete protein, as part of a fusion protein, or embedded in a protein chimera (depending on the recombinant nucleic acid molecule or expression cassette used) is an antigen or comprises an antigen. In other words, in some embodiments, the immunogenic composition comprises a recombinant bacterium which comprises a recombinant nucleic acid molecule or expression cassette encoding a polypeptide that comprises an antigen. Suitable antigens include, but are not limited to, any of those described herein (e.g., above in Section IV). In some embodiments, the recombinant bacterium in the immunogenic composition expresses the polypeptide comprising the antigen at a level sufficient to induce an immune response to the antigen upon administration of the composition to a host (e.g., a mammal such as a human). In some embodiments, the immune response stimulated by the immunogenic composition is a cell-mediated immune response. In some embodiments, the immune response stimulated by the immunogenic composition is a humoral immune response. In some embodiments, the immune response stimulated by the immunogenic composition comprises both a humoral and cell-mediated immune response.

[0344] For instance, in one aspect, the invention provides an immunogenic composition comprising a recombinant bacterium, where the bacterium comprises an expression cassette comprising the following: (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium; (b) a second polynucleotide encoding an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0345] In another aspect, the invention provides an immunogenic composition comprising a recombinant bacterium, where the bacterium comprises an expression cassette that comprises the following: (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; (b) a second polynucleotide encoding an antigen in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0346] In still another aspect, the invention provides an immunogenic composition comprising a recombinant *Listeria* bacterium, wherein the recombinant *Listeria* bacterium comprises an expression cassette, wherein the expression cassette comprises the following: (a) a polynucleotide that encodes a non-*Listerial* antigen and that is codon-optimized for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the antigen.

[0347] The invention also provides an immunogenic composition comprising a recombinant *Listeria* bacterium comprising an expression cassette which comprises: (a) a first polynucleotide encoding a non-*Listerial* signal peptide; (b) a second polynucleotide encoding an antigen that is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to both the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising both the non-*Listerial* signal peptide and the antigen.

[0348] In another aspect, the invention provides an immunogenic composition (or vaccine) comprising a recombinant bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, and (b) a second polynucleotide encoding a polypeptide comprising an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide.

[0349] In another aspect, the invention provides an immunogenic composition (or vaccine) comprising a recombinant *Listeria* bacterium, wherein the recombinant bacterium comprises a recombinant nucleic acid molecule which comprises (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in *Listeria*, and (b) a second polynucleotide encoding a polypeptide comprising an antigen, wherein the second polynucleotide is in the same translational reading

frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide.

[0350] In another aspect, the invention provides an immunogenic composition (or vaccine) comprising a recombinant bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a first polynucleotide encoding a non-secA1 bacterial signal peptide, and a second polynucleotide encoding a polypeptide comprising an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide.

[0351] In still another aspect, the invention provides an immunogenic composition (or vaccine) comprising a recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide, and (b) a second polynucleotide encoding a polypeptide either heterologous to the signal peptide or foreign to the bacterium, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide. In some embodiments, the polypeptide encoded by the first polynucleotide comprises an antigen.

[0352] In another aspect, the invention provides an immunogenic composition (or vaccine) comprising a recombinant *Listeria* bacterium, wherein the recombinant *Listeria* bacterium comprises a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a polynucleotide encoding a polypeptide foreign to *Listeria*, wherein the polynucleotide encoding the foreign polypeptide is codon-optimized for expression in *Listeria*. In some embodiments, the foreign polypeptide comprises an antigen.

[0353] In another aspect, the invention provides an immunogenic composition (or vaccine) comprising a recombinant *Listeria* bacterium, wherein the recombinant bacterium comprises a recombinant nucleic acid molecule comprising (a) a first polynucleotide encoding a non-*Listeria* signal peptide, and (b) a second polynucleotide encoding a polypeptide comprising an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-*Listeria* signal peptide and the polypeptide.

[0354] The invention also provides an immunogenic composition (or vaccine) comprising a recombinant bacterium, wherein the recombinant bacterium comprises a nucleic acid molecule comprising (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the polypeptide encoded by the second polynucleotide and the autolysin, or catalytically active fragment or catalytically active variant thereof, wherein, in the protein chimera, the polypeptide is fused to or is positioned within the autolysin,

or catalytically active fragment or catalytically active variant thereof. In some embodiments, the polypeptide encoded by the second polynucleotide comprises an antigen.

[0355] In another aspect, the invention provides an immunogenic composition (or vaccine) comprising a recombinant *Listeria* bacterium, wherein the recombinant *Listeria* bacterium comprises a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule encodes at least two discrete non-*Listeria* polypeptides, at least one of which comprises an antigen.

[0356] In other aspects, the invention provides an immunogenic composition (or vaccine) comprising a recombinant bacterium, which comprises a recombinant nucleic acid molecule comprising (a) a first polynucleotide encoding a signal peptide, (b) a second polynucleotide encoding a secreted protein, or a fragment thereof, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and (c) a third polynucleotide encoding a polypeptide heterologous to the secreted protein, or fragment thereof, wherein the third polynucleotide is in the same translational reading frame as the first and second polynucleotides, wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the third polynucleotide, and the secreted protein, or fragment thereof, and wherein the polypeptide encoded by the third polynucleotide is fused to the secreted protein, or fragment thereof, or is positioned within the secreted protein, or fragment thereof, in the protein chimera. In some embodiments, the heterologous polypeptide encoded by the third polynucleotide comprises an antigen.

[0357] It can be determined if a particular form of recombinant bacteria (and/or a particular expression cassette) is useful in an immunogenic composition (or as a vaccine) by testing the ability of the recombinant bacteria to stimulate an immune response in vitro or in a model system.

[0358] These immune cell responses can be measured by both in vitro and in vivo methods to determine if the immune response of a particular recombinant bacterium (and/or a particular expression cassette) is effective. One possibility is to measure the presentation of the protein or antigen of interest by an antigen-presenting cell that has been mixed with a population of the recombinant bacteria. The recombinant bacteria may be mixed with a suitable antigen presenting cell or cell line, for example a dendritic cell, and the antigen presentation by the dendritic cell to a T cell that recognizes the protein or antigen can be measured. If the recombinant bacteria are expressing the protein or antigen at a sufficient level, it will be processed into peptide fragments by the dendritic cells and presented in the context of MHC class I or class II to T cells. For the purpose of detecting the presented protein or antigen, a T cell clone or T cell line responsive to the particular protein or antigen may be used. The T cell may also be a T cell hybridoma, where the T cell is immortalized by fusion with a cancer cell line. Such T cell hybridomas, T cell clones, or T cell lines can comprise either CD8+ or CD4+ T cells. The dendritic cell can present to either CD8+ or CD4+ T cells, depending on the pathway by which the antigens are processed. CD8+ T cells recognize antigens in the context of MHC class I while CD4+ recognize antigens in the context of MHC class II. The T cell will be stimulated by the presented antigen through specific

recognition by its T cell receptor, resulting in the production of certain proteins, such as IL-2, tumor necrosis factor- α (TNF- α), or interferon- γ (IFN- γ), that can be quantitatively measured (for example, using an ELISA assay, ELISPOT assay, or Intracellular Cytokine Staining (ICS)). These are techniques that are well known in the art and that are also exemplified below in the Examples (see, e.g., Example 21, below).

[0359] Alternatively, a hybridoma can be designed to include a reporter gene, such as β -galactosidase, that is activated upon stimulation of the T cell hybridoma by the presented antigens. The increase in the production of β -galactosidase can be readily measured by its activity on a substrate, such as chlorophenol red-B-galactoside, which results in a color change. The color change can be directly measured as an indicator of specific antigen presentation.

[0360] Additional in vitro and in vivo methods for assessing the antigen expression of recombinant bacteria vaccines of the present invention are known to those of ordinary skill in the art. It is also possible to directly measure the expression of a particular heterologous antigen by recombinant bacteria. For example, a radioactively labeled amino acid can be added to a cell population and the amount of radioactivity incorporated into a particular protein can be determined. The proteins synthesized by the cell population can be isolated, for example by gel electrophoresis or capillary electrophoresis, and the amount of radioactivity can be quantitatively measured to assess the expression level of the particular protein. Alternatively, the proteins can be expressed without radioactivity and visualized by various methods, such as an ELISA assay or by gel electrophoresis and Western blot with detection using an enzyme linked antibody or fluorescently labeled antibody.

[0361] Example 21, below, provides some specific exemplary examples of how some of the techniques known to those of ordinary skill in the art can be used to assess immunogenicity. For instance, Elispot assay, Intracellular Cytokine Staining Assay (ICS), measurement of cytokine expression of stimulated spleen cells, and assessment of cytotoxic T cell activity in vitro and in vivo are all techniques for assessing immunogenicity known to those in the art. Exemplary descriptions of these techniques with model antigens are provided in Example 21. Exemplary assays are also described in Examples 31A and 31E, below.

[0362] In addition, therapeutic efficacy of the vaccine composition can be assessed more directly by administration of the immunogenic composition or vaccine to an animal model such as a mouse model, followed by an assessment of survival or tumor growth. For instance, survival can be measured following administration and challenge (e.g., with a tumor or pathogen). See, e.g., the assays described in Examples 20 and 31B-D, below.)

[0363] Mouse models useful for testing the immunogenicity of an immunogenic composition or vaccine expressing a particular antigen can be produced by first modifying a tumor cell so that it expresses the antigen of interest or a model antigen and then implanting the tumor cells expressing the antigen of interest into mice. The mice can be vaccinated with the candidate immunogenic composition or vaccine comprising a recombinant bacterium expressing a polypeptide comprising the antigen of interest or a model antigen prior to implantation of the tumor cells (to test

prophylactic efficacy of the candidate composition) or following implantation of the tumor cells in the mice (to test therapeutic efficacy of the candidate composition).

[0364] As an example, CT26 mouse murine colon carcinoma cells can be transfected with an appropriate vector comprising an expression cassette encoding the desired antigen or model antigen using techniques standard in the art. Standard techniques such as flow cytometry and Western blots can then be used to identify clones expressing the antigen or model antigen at sufficient levels for use in the immunogenicity and/or efficacy assays.

[0365] Alternatively, candidate compositions can be tested which comprise a recombinant bacterium expressing an antigen that corresponds to or is derived from an antigen endogenous to a tumor cell line (e.g., the retroviral gp70 tumor antigen AH1 endogenous to CT26 mouse murine colon carcinoma cells, or the heteroclitic epitope AH1-A5). In such assays, the tumor cells can be implanted in the animal model without further modification to express an additional antigen. Candidate vaccines comprising the antigen can then be tested.

[0366] As indicated, vaccine compositions comprising the bacteria described herein are also provided. For instance, the invention provides a vaccine comprising a recombinant bacterium described herein (see, e.g., the recombinant bacteria described above in the Summary of the Invention, Sections I and VIII of the Detailed Description above, and elsewhere in the specification, including the Examples, below) where the polypeptide sequence that is part of the polypeptide expressed by the recombinant bacterium as a discrete protein, as part of a fusion protein, or embedded in a protein chimera (depending on the recombinant nucleic acid molecule or expression cassette used) is an antigen. Suitable antigens include any of those described herein (e.g., above in Section IV).

[0367] In one aspect, the invention provides a vaccine that comprises a bacterium, wherein the bacterium comprises an expression cassette comprising the following: (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium; (b) a second polynucleotide encoding an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0368] In another aspect, the invention provides a vaccine that comprises a bacterium, where the bacterium comprises an expression cassette that comprises the following: (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; (b) a second polynucleotide encoding an antigen in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0369] In still another aspect, the invention provides a vaccine that comprises a recombinant *Listeria* bacterium comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises the following: (a) a polynucleotide that encodes a non-*Listerial* antigen and that is codon-optimized

for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the antigen.

[0370] In another aspect, the invention provides a vaccine comprising a recombinant *Listeria* bacterium comprising an expression cassette which comprises: (a) a first polynucleotide encoding a non-*Listerial* signal peptide; (b) a second polynucleotide encoding an antigen that is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to both the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising both the non-*Listerial* signal peptide and the antigen.

[0371] In some embodiments, the vaccine compositions comprise antigen-presenting cells (APC) which have been infected with any of the recombinant bacteria described herein. In some embodiments the vaccine (or immunogenic or pharmaceutical composition) does not comprise antigen-presenting cells (i.e., the vaccine or composition is a bacteria-based vaccine or composition, not an APC-based vaccine or composition).

[0372] Methods of administration suitable for administration of vaccine compositions (and pharmaceutical and immunogenic compositions) are known in the art, and include oral, intravenous, intradermal, intraperitoneal, intramuscular, intralymphatic, intranasal and subcutaneous routes of administration.

[0373] Vaccine formulations are known in the art and in some embodiments may include numerous additives, such as preservatives (e.g., thimerosal, 2-phenyoxy ethanol), stabilizers, adjuvants (e.g. aluminum hydroxide, aluminum phosphate, cytokines), antibiotics (e.g., neomycin, streptomycin), and other substances. In some embodiments, stabilizers, such as lactose or monosodium glutamate (MSG), are added to stabilize the vaccine formulation against a variety of conditions, such as temperature variations or a freeze-drying process. In some embodiments, vaccine formulations may also include a suspending fluid or diluent such as sterile water, saline, or isotonic buffered saline (e.g., phosphate buffered to physiological pH). Vaccine may also contain small amount of residual materials from the manufacturing process.

[0374] For instance, in some embodiments, the vaccine compositions are lyophilized (i.e., freeze-dried). The lyophilized preparation can be combined with a sterile solution (e.g., citrate-bicarbonate buffer, buffered water, 0.4% saline, or the like) prior to administration.

[0375] In some embodiments, the vaccine compositions may further comprise additional components known in the art to improve the immune response to a vaccine, such as adjuvants or co-stimulatory molecules. In addition to those listed above, possible adjuvants include chemokines and bacterial nucleic acid sequences, like CpG. In some embodiments, the vaccines comprise antibodies that improve the immune response to a vaccine, such as CTLA4. In some embodiments, co-stimulatory molecules comprise one or more factors selected from the group consisting of GM-CSF, IL-2, IL-12, IL-14, IL-15, IL-18, B7.1, B7.2, and B7-DC are optionally included in the vaccine compositions of the present invention. Other co-stimulatory molecules are known to those of ordinary skill in the art.

[0376] In additional aspects, the invention provides methods of improving a vaccine or immunogenic composition

comprising *Listeria* that express an antigen. Any of the polynucleotides, expression cassettes and/or expression vectors described herein may be used in these methods. For instance, the invention provides a method of improving a vaccine or immunogenic composition comprising a *Listeria* bacterium, wherein the *Listeria* bacterium expresses a heterologous antigen fused to a signal peptide, comprising codon-optimizing either the polypeptide-encoding sequence on the expression cassette, the signal peptide-encoding sequence of the expression cassette, or both. The invention provides a method of improving a vaccine or immunogenic composition comprising *Listeria* bacterium, wherein the *Listeria* bacterium expresses a heterologous antigen fused to a signal peptide, comprising using a signal peptide from a non-*Listerial* source and/or from a secretory pathway other than secA1.

[0377] Methods of producing the vaccines of the present invention are also provided. For instance, in one embodiment, a method of producing a vaccine comprising a recombinant bacterium (e.g. a recombinant *Listeria* bacterium) comprises introducing a recombinant nucleic acid molecule into the bacterium, expression cassette, or expression vector described herein into a bacterium, wherein the recombinant nucleic acid molecule, expression cassette, or expression vector encodes an antigen. For instance, in some embodiments, a recombinant nucleic acid molecule comprising (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium, and (b) a second polynucleotide encoding an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the antigen, is introduced into a bacterium to produce the vaccine. In some embodiments, a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide, and (b) a second polynucleotide encoding an antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the antigen, is introduced into the bacterium to produce the vaccine. In some embodiments, the recombinant nucleic acid molecule that is introduced into the bacterium to produce the vaccine is a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises (a) a first polynucleotide encoding a non-*Listerial* signal peptide, and (b) a second polynucleotide encoding an antigen that is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-*Listerial* signal peptide and the antigen. The recombinant nucleic acid molecule used to produce the vaccine is, in some embodiments, a recombinant nucleic acid molecule, comprising (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof, and (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, wherein the recombinant nucleic acid molecule encodes a protein chimera in which the non-*Listerial* polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof, or is inserted within the autolysin, or catalytically

active fragment or catalytically active variant thereof. In some other embodiments, a method of producing a vaccine comprising a recombinant *Listeria* bacterium is provided, which comprises introducing a polycistronic expression cassette, wherein the polycistronic expression cassette encodes at least two discrete non-*Listeria* polypeptides, where at least one of the polypeptides is an antigen, into a *Listeria* bacterium to produce vaccine.

[0378] Kits comprising any of the recombinant nucleic acid molecules, expression cassettes, vectors, bacteria and/or compositions of the invention are also provided.

[0379] X. Methods of Use

[0380] A variety of methods of using the recombinant bacteria or pharmaceutical, immunogenic, or vaccine compositions described herein for inducing immune responses, and/or preventing or treating conditions in a host are provided. In some embodiments, the condition that is treated or prevented is a disease. In some embodiments, the disease is cancer. In some embodiments, the disease is an infectious disease. In addition, the recombinant bacteria are also useful in the production and isolation of heterologous proteins, such as mammalian proteins.

[0381] As used herein, "treatment" or "treating" (with respect to a condition or a disease) is an approach for obtaining beneficial or desired results including and preferably clinical results. For purposes of this invention, beneficial or desired results with respect to a disease include, but are not limited to, one or more of the following: improving a condition associated with a disease, curing a disease, lessening severity of a disease, delaying progression of a disease, alleviating one or more symptoms associated with a disease, increasing the quality of life of one suffering from a disease, and/or prolonging survival. Likewise, for purposes of this invention, beneficial or desired results with respect to a condition include, but are not limited to, one or more of the following: improving a condition, curing a condition, lessening severity of a condition, delaying progression of a condition, alleviating one or more symptoms associated with a condition, increasing the quality of life of one suffering from a condition, and/or prolonging survival. For instance, in those embodiments where the compositions described herein are used for treatment of cancer, the beneficial or desired results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) neoplastic or cancerous cells, reducing metastasis of neoplastic cells found in cancers, shrinking the size of a tumor, decreasing symptoms resulting from the cancer, increasing the quality of life of those suffering from the cancer, decreasing the dose of other medications required to treat the disease, delaying the progression of the cancer, and/or prolonging survival of patients having cancer.

[0382] As used herein, the terms "preventing" disease or "protecting a host" from disease (used interchangeably herein) encompass, but are not limited to, one or more of the following: stopping, deferring, hindering, slowing, retarding, and/or postponing the onset or progression of a disease, stabilizing the progression of a disease, and/or delaying development of a disease. The terms "preventing" a condition or "protecting a host" from a condition (used interchangeably herein) encompass, but are not limited to, one or more of the following: stopping, deferring, hindering, slowing, retarding, and/or postponing the onset or progression of

a condition, stabilizing the progression of a condition, and/or delaying development of a condition. The period of this prevention can be of varying lengths of time, depending on the history of the disease or condition and/or individual being treated. By way of example, where the vaccine is designed to prevent or protect against an infectious disease caused by a pathogen, the terms "preventing" disease or "protecting a host" from disease encompass, but are not limited to, one or more of the following: stopping, deferring, hindering, slowing, retarding, and/or postponing the infection by a pathogen of a host, progression of an infection by a pathogen of a host, or the onset or progression of a disease associated with infection of a host by a pathogen, and/or stabilizing the progression of a disease associated with infection of a host by a pathogen. Also, by way of example, where the vaccine is an anti-cancer vaccine, the terms "preventing" disease or "protecting the host" from disease encompass, but are not limited to, one or more of the following: stopping, deferring, hindering, slowing, retarding, and/or postponing the development of cancer or metastasis, progression of a cancer, or a reoccurrence of a cancer.

[0383] In one aspect, the invention provides a method of inducing an immune response in a host to an antigen, comprising administering to the host an effective amount of a recombinant bacterium described herein or an effective amount of a composition (e.g., a pharmaceutical composition, immunogenic composition, or vaccine) comprising a recombinant bacterium described herein (see, e.g., the Summary of the Invention, Sections I, VIII, and IX of the Detailed Description above, or the Examples below). In some embodiments, the polypeptide encoded by the recombinant nucleic acid, expression cassette, and/or expression vector in the recombinant bacterium comprises the antigen or is a fusion protein or protein chimera comprising the antigen.

[0384] For instance, in one aspect, the invention provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising a recombinant bacterium, wherein the recombinant bacterium comprises an expression cassette comprising the following: (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in the bacterium; (b) a second polynucleotide encoding the antigen, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0385] In another aspect, the invention provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising a recombinant bacterium comprising an expression cassette, where the expression cassette comprises the following: (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; (b) a second polynucleotide encoding the antigen in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0386] In yet another aspect, the invention provides a method of inducing an immune response in a host to a non-*Listeria* antigen comprising administering to the host an effective amount of a composition comprising a recombinant *Listeria* bacterium comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises the following: (a) a polynucleotide which encodes the non-*Listeria* antigen and that is codon-optimized for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the antigen.

[0387] In another aspect, the invention provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a recombinant *Listeria* bacterium comprising an expression cassette which comprises the following: (a) a first polynucleotide encoding a non-*Listeria* signal peptide; (b) a second polynucleotide encoding the antigen that is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to both the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising both the non-*Listeria* signal peptide and the polypeptide.

[0388] In some embodiments of the methods of inducing immune responses described herein, the bacterium is administered in the form of a pharmaceutical composition, an immunogenic composition and/or vaccine composition.

[0389] In some embodiments, the immune response is an MHC Class I immune response. In other embodiments, the immune response is an MHC Class II immune response. In still other embodiments, the immune response that is induced by administration of the bacteria or compositions is both an MHC Class I and an MHC Class II response. Accordingly, in some embodiments, the immune response comprises a CD4+ T-cell response. In some embodiments, the immune response comprises a CD8+ T-cell response. In some embodiments, the immune response comprises both a CD4+ T-cell response and a CD8+ T-cell response. In some embodiments, the immune response comprises a B-cell response and/or a T-cell response. B-cell responses may be measured by determining the titer of an antibody directed against the antigen, using methods known to those of ordinary skill in the art. In some embodiments, the immune response which is induced by the compositions described herein is a humoral response. In other embodiments, the immune response which is induced is a cellular immune response. In some embodiments, the immune response comprises both cellular and humoral immune responses. In some embodiments, the immune response is antigen-specific. In some embodiments, the immune response is an antigen-specific T-cell response.

[0390] In addition to providing methods of inducing immune responses, the present invention also provides methods of preventing or treating a condition in a host (e.g., a subject such as human patient). In some embodiments, the condition is a disease. The methods comprise administration to the host of an effective amount of a recombinant bacterium described herein, or a composition comprising a recombinant bacterium described herein (see, e.g., the Summary of the Invention, Sections I, VIII, and IX of the Detailed Description above, or the Examples below). In some embodiments, the disease is cancer. In some embodiments, the disease is an infectious disease.

[0391] For instance, in one aspect, the invention provides a method of preventing or treating disease (or condition) in a host comprising administering to the host an effective amount of a composition comprising a bacterium, wherein the bacterium comprises an expression cassette comprising the following: (a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a bacterium; (b) a second polynucleotide encoding a polypeptide (e.g., an antigen and/or a therapeutic mammalian protein), wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0392] In another aspect, the invention provides a method of preventing or treating disease (or condition) in a host comprising administering to the host an effective amount of a composition comprising a recombinant bacterium, where the bacterium comprises an expression cassette, and where the expression cassette comprises the following: (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; (b) a second polynucleotide encoding a polypeptide (e.g., an antigen and/or mammalian protein) in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to the first and second polynucleotides, so that the expression cassette encodes a fusion protein comprising the signal peptide and the antigen.

[0393] In still another aspect, the invention provides a method of preventing or treating disease (or a condition) in a host comprising administering to the host an effective amount of a composition comprising a recombinant *Listeria* bacterium comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises the following: (a) a polynucleotide which encodes a non-*Listeria* polypeptide (e.g., an antigen and/or a therapeutic mammalian protein) and that is codon-optimized for expression in *Listeria*; and (b) a promoter, operably linked to the polynucleotide encoding the antigen.

[0394] In another aspect, the invention provides a method of preventing or treating disease (or a condition) in a host comprising administering to the host an effective amount of a composition comprising a recombinant *Listeria* bacterium comprising an expression cassette which comprises: (a) a first polynucleotide encoding a non-*Listeria* signal peptide; (b) a second polynucleotide encoding a polypeptide (e.g., an antigen and/or a therapeutic mammalian protein) that is in the same translational reading frame as the first polynucleotide; and (c) a promoter operably linked to both the first and second polynucleotides, wherein the expression cassette encodes a fusion protein comprising both the non-*Listeria* signal peptide and the polypeptide.

[0395] In some embodiments, the disease is cancer. In some embodiments, where the condition being treated or prevented is cancer, the disease is melanoma, breast cancer, pancreatic cancer, liver cancer, colon cancer, colorectal cancer, lung cancer, brain cancer, testicular cancer, ovarian cancer, squamous cell cancer, gastrointestinal cancer, cervical cancer, kidney cancer, thyroid cancer or prostate cancer. In some embodiments, the cancer is melanoma. In some embodiments, the cancer is pancreatic cancer. In some

embodiments, the cancer is colon cancer. In some embodiments, the cancer is prostate cancer. In some embodiments, the cancer is metastatic.

[0396] In other embodiments, the disease is an autoimmune disease. In still other embodiments, the disease is an infectious disease or another disease caused by a pathogen such as a virus, bacterium, fungus, or protozoa. In some embodiments, the disease is an infectious disease.

[0397] In some embodiments, the use of the recombinant bacteria in the prophylaxis or treatment of a cancer comprises the delivery of the recombinant bacteria to cells of the immune system of an individual to prevent or treat a cancer present or to which the individual has increased risk factors, such as environmental exposure and/or familial disposition. In other embodiments, the use of the recombinant bacteria in the prophylaxis or treatment of a cancer comprises delivery of the recombinant bacteria to an individual who has had a tumor removed or has had cancer in the past, but is currently in remission.

[0398] In some embodiments, administration of composition comprising a recombinant bacterium described herein to a host elicits a CD4+ T-cell response in the host. In some other embodiments, administration of a composition comprising a recombinant bacterium described herein to a host elicits a CD8+ T-cell response in the host. In some embodiments, administration of a composition comprising a recombinant bacterium described herein elicits both a CD4+ T-cell response and a CD8+ T-cell response in the host.

[0399] The efficacy of the vaccines or other compositions for the treatment of a condition can be evaluated in an individual, for example in mice. A mouse model is recognized as a model for efficacy in humans and is useful in assessing and defining the vaccines of the present invention. The mouse model is used to demonstrate the potential for the effectiveness of the vaccines in any individual. Vaccines can be evaluated for their ability to provide either a prophylactic or therapeutic effect against a particular disease. For example, in the case of infectious diseases, a population of mice can be vaccinated with a desired amount of the appropriate vaccine of the invention, where the recombinant bacterium expresses an infectious disease associated antigen. The mice can be subsequently infected with the infectious agent related to the vaccine antigen and assessed for protection against infection. The progression of the infectious disease can be observed relative to a control population (either non vaccinated or vaccinated with vehicle only or a bacterium that does not contain the appropriate antigen).

[0400] In the case of cancer vaccines, tumor cell models are available, where a tumor cell line expressing a desired tumor antigen can be injected into a population of mice either before (therapeutic model) or after (prophylactic model) vaccination with a composition comprising a bacterium of the invention containing the desired tumor antigen. Vaccination with a recombinant bacterium containing the tumor antigen can be compared to control populations that are either not vaccinated, vaccinated with vehicle, or with a recombinant bacterium that expresses an irrelevant antigen. The effectiveness of the vaccine in such models can be evaluated in terms of tumor volume as a function of time after tumor injection or in terms of survival populations as a function of time after tumor injection (e.g., Example 31D). In one embodiment, the tumor volume in mice vaccinated

with a composition comprising the recombinant bacterium is about 5%, about 10%, about 25%, about 50%, about 75%, about 90% or about 100% less than the tumor volume in mice that are either not vaccinated or are vaccinated with vehicle or a bacterium that expresses an irrelevant antigen. In another embodiment, this differential in tumor volume is observed at least about 10, about 17, or about 24 days following the implant of the tumors into the mice. In one embodiment, the median survival time in the mice vaccinated with the composition comprising a recombinant bacterium is at least about 2, about 5, about 7 or at least about 10 days longer than in mice that are either not vaccinated or are vaccinated with vehicle or bacteria that express an irrelevant antigen.

[0401] The host (i.e., subject) in the methods described herein, is any vertebrate, preferably a mammal, including domestic animals, sport animals, and primates, including humans. In some embodiments, the host is a mammal. In some embodiments, the host is a human.

[0402] The delivery of the recombinant bacteria, or a composition comprising the strain, may be by any suitable method, such as intradermal, subcutaneous, intraperitoneal, intravenous, intramuscular, intralymphatic, oral or intranasal, as well as by any route that is relevant for any given malignant or infectious disease or other condition.

[0403] The compositions comprising the recombinant bacteria and an immunostimulatory agent may be administered to a host simultaneously, sequentially or separately. Examples of immunostimulatory agents include, but are not limited to IL-2, IL-12, GM-CSF, IL-15, B7.1, B7.2, and B7-DC and IL-14. Additional examples of stimulatory agents are provided in Section IX, above.

[0404] As used herein, an "effective amount" of a bacterium or composition (such as a pharmaceutical composition or an immunogenic composition) is an amount sufficient to effect beneficial or desired results. For prophylactic use, beneficial or desired results includes results such as eliminating or reducing the risk, lessening the severity, or delaying the outset of the disease, including biochemical, histologic and/or behavioral symptoms of a disease, its complications and intermediate pathological phenotypes presenting during development of the disease. For therapeutic use, beneficial or desired results includes clinical results such as inhibiting or suppressing a disease, decreasing one or more symptoms resulting from a disease (biochemical, histologic and/or behavioral), including its complications and intermediate pathological phenotypes presenting during development of a disease, increasing the quality of life of those suffering from a disease, decreasing the dose of other medications required to treat the disease, enhancing effect of another medication, delaying the progression of the disease, and/or prolonging survival of patients. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of drug, compound, or pharmaceutical composition is an amount sufficient to accomplish prophylactic or therapeutic treatment either directly or indirectly. As is understood in the clinical context, an effective amount of a drug, compound, or pharmaceutical composition may or may not be achieved in conjunction with another drug, compound, or pharmaceutical composition. Thus, an effective amount may be considered in the context of administering one or more

therapeutic agents, and a single agent may be considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable result may be or is achieved.

[0405] In some embodiments, for a therapeutic treatment of a cancer, an effective amount includes an amount that will result in the desired immune response, wherein the immune response either slows the growth of the targeted tumors, reduces the size of the tumors, or preferably eliminates the tumors completely. The administration of the vaccine may be repeated at appropriate intervals, and may be administered simultaneously at multiple distinct sites in the vaccinated individual. In some embodiments, for a prophylactic treatment of a cancer, an effective amount includes a dose that will result in a protective immune response such that the likelihood of an individual to develop the cancer is significantly reduced. The vaccination regimen may be comprised of a single dose, or may be repeated at suitable intervals until a protective immune response is established.

[0406] In some embodiments, the therapeutic treatment of an individual for cancer may be started on an individual who has been diagnosed with a cancer as an initial treatment, or may be used in combination with other treatments. For example, individuals who have had tumors surgically removed or who have been treated with radiation therapy or by chemotherapy may be treated with the vaccine in order to reduce or eliminate any residual tumors in the individual, or to reduce the risk of a recurrence of the cancer. In some embodiments, the prophylactic treatment of an individual for cancer, would be started on an individual who has an increased risk of contracting certain cancers, either due to environmental conditions or genetic predisposition.

[0407] The dosage of the pharmaceutical compositions or vaccines that are given to the host will vary depending on the species of the host, the size of the host, and the condition or disease of the host. The dosage of the compositions will also depend on the frequency of administration of the compositions and the route of administration. The exact dosage is chosen by the individual physician in view of the patient to be treated.

[0408] In some embodiments, the pharmaceutical compositions, immunogenic compositions, or vaccines used in the methods comprise recombinant bacteria which comprise the recombinant nucleic acid molecules, expression cassettes and/or expression vectors described herein. In some embodiments, the recombinant bacteria are modified and/or mutant bacteria such as those described in U.S. patent application Ser. No. 10/883,599, entitled "Modified Free-Living Microbes, Vaccine Compositions and Methods of Use Thereof," by Thomas W. Dubensky, Jr. et al., filed Jun. 30, 2004, U.S. patent Publication No. 2004/0228877 and U.S. patent Publication No. 2004/0197343, each of which is incorporated by reference herein in its entirety. In some embodiments, a single dose of the pharmaceutical composition or vaccine comprising such modified and/or mutant bacteria or any of the other recombinant bacteria described herein comprises from about 10^2 to about 10^{12} of the bacterial organisms. In another embodiment, a single dose comprises from about 10^5 to about 10^{11} of the bacterial organisms. In another embodiment, a single dose comprises from about 10^6 to about 10^{11} of the bacterial organisms. In still another embodiment, a single dose of the pharmaceu-

tical composition or vaccine comprises from about 10^7 to about 10^{10} of the bacterial organisms. In still another embodiment, a single dose of the pharmaceutical composition or vaccine comprises from about 10^7 to about 10^9 of the bacterial organisms.

[0409] In some embodiments, a single dosage comprises at least about 1×10^2 bacterial organisms. In some embodiments, a single dose of the composition comprises at least about 1×10^5 organisms. In another embodiment, a single dose of the composition or vaccine comprises at least about 1×10^6 bacterial organisms. In still another embodiment, a single dose of the composition or vaccine comprises at least about 1×10^7 of the bacterial organisms.

[0410] In some embodiments, a single dose of the pharmaceutical composition, immunogenic composition, or vaccine comprising recombinant, modified and/or mutant bacteria described herein comprises from about 1 CFU/kg to about 1×10^{10} CFU/kg (CFU=colony forming units). In some embodiments, a single dose of the composition comprises from about 10 CFU/kg to about 1×10^9 CFU/kg. In another embodiment, a single dose of the composition or vaccine comprises from about 1×10^2 CFU/kg to about 1×10^8 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises from about 1×10^3 CFU/kg to about 1×10^8 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises from about 1×10^4 CFU/kg to about 1×10^7 CFU/kg. In some embodiments, a single dose of the composition comprises at least about 1 CFU/kg. In some embodiments, a single dose of the composition comprises at least about 10 CFU/kg. In another embodiment, a single dose of the composition or vaccine comprises at least about 1×10^2 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises at least about 1×10^3 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises from at least about 1×10^4 CFU/kg.

[0411] In some embodiments, the proper (i.e., effective) dosage amount for one host, such as human, may be extrapolated from the LD₅₀ data for another host, such as a mouse, using methods known to those in the art.

[0412] In some embodiments, the pharmaceutical composition, immunogenic composition, or vaccine comprises antigen-presenting cells, such as dendritic cells, which have been infected with recombinant bacteria comprising the recombinant nucleic acid molecules, expression cassettes and/or expression vectors described herein. In some embodiments, the bacteria have been modified and/or are mutants such as those described in U.S. patent application Ser. No. 10/883,599, filed Jun. 30, 2004, and U.S. patent Publication Nos. 2004/0228877 and US 2004/0197343, each of which is incorporated by reference herein in its entirety. Such antigen-presenting cell based vaccines are described, for instance, in the following: International Application No. PCT/US2004/23881, entitled "Antigen-Presenting Cell Vaccines and Methods of Use Thereof," by Thomas W. Dubensky, Jr. et al., filed Jul. 23, 2004; U.S. patent application Ser. No. 10/883,599, filed Jun. 30, 2004; U.S. patent Publication No. 2004/0228877; and U.S. patent Publication No. US 2004/0197343, each of which is incorporated by reference herein in its entirety. In some embodiments, an individual dosage of an antigen-presenting cell based vaccine comprising bacteria such as those described herein comprises

between about 1×10^3 to about 1×10^{10} antigen-presenting cells. In some embodiments, an individual dosage of the vaccine comprises between about 1×10^5 to about 1×10^9 antigen-presenting cells. In some embodiments, an individual dosage of the vaccine comprises between about 1×10^7 to about 1×10^9 antigen-presenting cells.

[0413] In some embodiments, multiple administrations of the dosage unit are preferred, either in a single day or over the course of a week or month or year or years. In some embodiments, the dosage unit is administered every day for multiple days, or once a week for multiple weeks.

[0414] The invention further provides the use of any recombinant bacterium described herein (i.e., any bacterium comprising a recombinant nucleic acid molecule, expression cassette, or vector described herein) in the manufacture of a medicament for inducing an immune response in a host to an antigen, wherein a polypeptide encoded by the recombinant nucleic acid molecule, expression cassette, and/or vector in the bacterium comprises the antigen. In some embodiments, the antigen is a heterologous antigen. The invention also provides the use of a recombinant bacterium described herein in the manufacture of a medicament for preventing or treating a condition in a host (e.g., a disease such as cancer or an infectious disease). The invention further provides the recombinant bacteria described herein for use in inducing an immune response in a host to an antigen, wherein a polypeptide encoded by the recombinant nucleic acid molecule, expression cassette, and/or vector in the bacterium comprises the antigen. The invention further provides the recombinant bacteria described herein for use in the prevention or treatment of a condition (such as a disease) in a host.

[0415] The invention also provides a method of inducing MHC class I antigen presentation or MHC class II antigen presentation on an antigen-presenting cell comprising contacting a bacterium described herein with an antigen-presenting cell.

[0416] The invention further provides a method of inducing an immune response in a host to an antigen comprising, the following steps: (a) contacting a recombinant bacterium described herein with an antigen-presenting cell from the host, under suitable conditions and for a time sufficient to load the antigen-presenting cells; and (b) administering the antigen-presenting cell to the host.

[0417] Other possible uses of the recombinant nucleic acid molecules, expression cassettes, and bacteria will be recognized by those of ordinary skill in the art. For instance, the recombinant nucleic acid molecules, expression cassettes, vectors, and recombinant bacteria (and other host cells) described herein are useful for the production and isolation of heterologous polypeptides. Accordingly in an alternative aspect, the invention provides a method of expressing a polypeptide in a bacterium, comprising (a) introducing an expression cassette or vector described herein into bacteria (e.g., via transfection, transformation, or conjugation); and (b) growing the bacteria in culture under conditions suitable for protein expression. In another alternative aspect, the invention provides a method of producing an isolated polypeptide comprising the following: (a) introducing an expression cassette or vector described herein into bacteria (e.g., via transfection, transformation, or conjugation); (b) growing the bacteria in cell culture under conditions suitable for protein expression; and (c) isolating the protein from the

bacterial cell culture. Suitable methods of transformation, transfection, and conjugation are well known to those of ordinary skill in the art, as are methods of culturing and growing bacteria and isolating secreted or non-secreted protein from cell culture.

EXAMPLES

[0418] The following examples are provided to illustrate, but not to limit, the invention.

Example 1

Preparation of Exemplary Mutant *Listeria* strains

[0419] *Listeria* strains were derived from 10403S (Bishop et al., *J. Immunol.* 139:2005 (1987)). *Listeria* strains with in-frame deletions of the indicated genes were generated by SOE-PCR and allelic exchange with established methods (Camilli, et al., *Mol. Microbiol.* 8:143 (1993)). The mutant strain LLO L461T (DP-L4017) was described in Glomski, et al., *J. Cell. Biol.* 156: 1029 (2002), incorporated by reference herein. The actA⁻ mutant (DP-L4029) is the DP-L3078 strain described in Skoble et al., *J. of Cell Biology*, 150: 527-537 (2000), incorporated by reference herein in its entirety, which has been cured of its prophage. (Prophage curing is described in (Lauer et al., *J. Bacteriol.* 184:4177 (2002); U.S. patent Publication No. 2003/0203472).) Construction of an actA⁻/uvrAB⁻ strain is described in the U.S. provisional application 60/446,051, filed Feb. 6, 2003, as L4029/uvrAB (see, e.g. Example 7 of that application), as well as in U.S. patent Publication No. 2004/0197343. DP-L4029uvrAB (a *Listeria monocytogenes* actA⁻/uvrAB⁻ double deletion mutant) was deposited with the American Type Culture Collection (ATCC), at 10801 University Blvd, Manassas, Va., 20110-2209, United States of America, on Oct. 3, 2003, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and designated PTA-5563. Additional descriptions regarding mutant *Listeria* are provided in the following applications or publications, each of which is incorporated by reference herein in its entirety: U.S. patent Publication No. 2004/0228877; U.S. patent Publication No. US 2004/0197343; the PCT International Application No. PCT/US2004/23881, filed Jul. 23, 2004; and U.S. patent application Ser. No. 10/883,599, filed Jun. 30, 2004. In addition, an exemplary *Listeria monocytogenes* Δ actA Δ inlB double deletion mutant has been deposited with the American Type Culture Collection (ATCC), at 10801 University Blvd, Manassas, Va., 20110-2209, United States of America, on Oct. 3, 2003, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and designated PTA-5562.

[0420] One non-limiting example of a method of deleting a gene in *Listeria monocytogenes* to generate an attenuated mutant is provided in Example 24, below.

Example 2

Construction of *Listeria* strains expressing AH1/OVA or AH1-A5/OVA

[0421] Mutant *Listeria* strains expressing a truncated form of a model antigen ovalbumin (OVA), the immunodominant epitope from mouse colorectal cancer (CT26) known as

AH1 (SPSYVYHQF (SEQ ID NO:72)), and the altered epitope AH1-A5 (SPSYAYHQF (SEQ ID NO:73); Slansky et al., *Immunity*, 13:529-538 (2000)) were prepared. The pPL2 integrational vector (Lauer et al., *J. Bacteriol.* 184:4177 (2002); U.S. patent Publication No. 2003/0203472) was used to derive OVA and AH1-A5/OVA recombinant *Listeria* strains containing a single copy integrated into an innocuous site of the *Listeria* genome.

[0422] A. Construction of OVA-Expressing *Listeria* (DP-L4056).

[0423] An antigen expression cassette consisting of hemolysin-deleted LLO fused with truncated OVA and contained in the pPL2 integration vector (pPL2/LLO-OVA) is first prepared. The *Listeria*-OVA vaccine strain is derived by introducing pPL2/LLO-OVA into the phage-cured *L. monocytogenes* strain DP-L4056 at the PSA (Phage from ScottA) attachment site TRNA^{As}-attBB'.

[0424] PCR is used to amplify the hemolysin-deleted LLO using the following template and primers:

[0425] Source: DP-L4056 genomic DNA

[0426] Primers:

Forward (KpnI-LLO nts. 1257-1276):
(SEQ ID NO:74)
5'-CTCTGGTACCTCCTTTGATTAGTATATTC
(T_m: LLO-spec: 52° C. Overall: 80° C.)
Reverse (BamHI-XhoI-LLO nts. 2811-2792):
(SEQ ID NO:75)
5'-CAATGGATCCCTCAGATCATAATTACTTCATCCC
(T_m: LLO-spec: 52° C. Overall: 102° C.)

[0427] PCR is also used to amplify the truncated OVA using the following template and primers:

[0428] Source: pDP3616 plasmid DNA from DP-E3616 *E. coli* (Higgins et al., *Mol. Molbiol.* 31:1631-1641 (1999)).

[0429] Primers:

Forward (XhoI- NcoI OVA cDNA nts. 174-186):
(SEQ ID NO:76)
5'-ATTTCTCGAGTCCATGGGGGTCTCATCATC
(T_m: OVA-spec: 60° C. Overall: 88° C.)
Reverse (XhoI-NotI-HindIII):
(SEQ ID NO:77)
5'-GGTGCTCGAGTCCGGCCGCAAGCTT
(T_m: Overall: 82° C.)

[0430] One protocol for completing the construction process involves first cutting the LLO amplicon with KpnI and BamHI and inserting the KpnI/BamHI vector into the pPL2 vector (pPL2-LLO). The OVA amplicon is then cut with XhoI and NotI and inserted into the pPL2-LLO which has been cut with XhoI/NotI. (Note: The pPL2 vector does not contain any XhoI sites; pDP-3616 contains one XhoI site, that is exploited in the OVA reverse primer design.) The construct pPL2/LLO-OVA is verified by restriction analysis (KpnI-LLO-XhoI-OVA-NotI) and sequencing. The plasmid pPL2/LLO-OVA is introduced into *E. coli* by transformation, followed by introduction and integration into *Listeria* (DP-L4056) by conjugation, exactly as described by Lauer

et al. (or into another desired strain of *Listeria*, such as an inlB⁻ mutant or an inlB⁻actA⁻ double mutant).

[0431] B. Construction of *Listeria* Strains Expressing AH1/OVA or AH1-A5/OVA.

[0432] To prepare *Listeria* expressing either the AH1/OVA or the AH1-A5/OVA antigen sequences, inserts bearing the antigen are first prepared from oligonucleotides and then ligated into the vector pPL2/LLO-OVA (prepared as described above).

[0433] The following oligonucleotides are used in preparation of the AH1 or AH1-A5 insert:

[0434] AH1 epitope insert (ClaI-PstI compatible ends):

Top strand oligo (AH1 Top):
(SEQ ID NO:78)
5'-CGATTCCCCTAGTTATGTTTACCACCAATTGCTGCA
Bottom strand oligo (AH1 Bottom):
(SEQ ID NO:79)
5'-GCAAATTGGTGGTAAACATAACTAGGGGAAT

[0435] AH1-A5 epitope insert (ClaI-AvaII compatible ends):

The sequence of the AH1-A5 epitope is SPSYAYHQF
(SEQ ID NO:73)
(5'-AGT CCA AGT TAT GCA TAT CAT CAA TTT-3'
(SEQ ID NO:80))

(SEQ ID NO:81)
Top: 5'-CGATAGTCCAAGTTATGCATATCATCAATTGTC
(SEQ ID NO:82)
Bottom: 5'-GTCGCAAATTGATGATATGCATAACTTGGACTAT

[0436] The oligonucleotide pair for a given epitope are mixed together at an equimolar ratio, heated at 95° C. for 5 min. The oligonucleotide mixture is then allowed to slowly cool. The annealed oligonucleotide pairs are then ligated at a 200 to 1 molar ratio with pPL2-LLO/OVA plasmid prepared by digestion with the relevant restriction enzymes. The identity of the new construct can be verified by restriction analysis and/or sequencing.

[0437] The plasmid can then be introduced into *E. coli* by transformation, followed by introduction and integration into *Listeria* (DP-L4056) by conjugation, exactly as described by Lauer et al., or into another desired strain of *Listeria*, such as an actA⁻ mutant strain (DP-L0429), LLO L461T strain (DP-L4017), or actA⁻/uvrAB⁻ strain (DP-L4029uvrAB).

Example 3

Construction of *Listeria* Polynucleotides and Expression Cassette Elements

[0438] A. Cloning Vectors

[0439] Selected heterologous antigen expression cassette molecular constructs were inserted into pPL2 (Lauer et al. *J. Bacteriol.* 2002), or pAM401 (Wirth et al., *J. Bacteriol.* 165:831-836), modified to contain the multiple cloning sequence of pPL2 (Aat II small fragment, 171 bps), inserted

between blunted Xba I and Nru I recognition sites, within the tetracycline resistance gene (pAM401-MCS, FIG. 32). In general, the hly promoter and (selected) signal peptide sequence was inserted between the unique Kpn I and Bam HI sites in the pPL2 or pAM401-MCS plasmid vectors. Selected EphA2 genes (sometimes modified to contain N-terminal and C-terminal epitope tags; see description below) were cloned subsequently into these constructs between unique Bam HI and Sac I sites. Molecular constructs based on the pAM401-MCS plasmid vector were introduced by electroporation into selected *Listeria monocytogenes* strains also treated with lysozyme, utilizing methods common to those skilled in the art. The expected plasmid structure in *Listeria*-transfectants was verified by isolating DNA from colonies that formed on chloramphenicol-containing BHI agar plates (10 µg/ml) by restriction enzyme analysis. Recombinant *Listeria* transformed with various pAM401-MCS based heterologous protein expression cassette constructs were utilized to measure heterologous protein expression and secretion, as described below.

[0440] The pPL2 based heterologous protein expression cassette constructs were incorporated into the tRNA^{Arg} gene in the genome of selected *Listeria* strains, according to the methods as described previously [Lauer et. al., J. Bacteriol. 184, 4177-4186 (2002)]. Briefly, the pPL2 heterologous protein expression cassette constructs plasmid was first introduced into the *E. coli* host strain SM10 (Simon et. al., Bio/Technology 1:784-791 (1983)) by electroporation or by chemical means. Subsequently, the pPL2-based plasmid was transferred from transformed SM10 to the selected *Listeria* strains by conjugation. Following incubation on drug-selective BHI agar plates containing 7.5 µg of chloramphenicol per ml and 200 µg of streptomycin per ml as described, selected colonies are purified by passaging 3 times on plates with the same composition. To verify integration of the pPL2 vector at the phage attachment site, individual colonies are picked and screened by PCR using the primer pair of forward primer NC 16 (5'-gtcaaacatacgctcttate-3' (SEQ ID NO:94)) and reverse primer PL95 (5'-acataatcagtcctcaagtagatgc-3' (SEQ ID NO:95)). Selected colonies having the pPL2-based plasmid incorporated into the tRNA^{Arg} gene in the genome of selected *Listeria* strains yielded a diagnostic DNA amplicon of 499 bps.

[0441] B. Promoter

[0442] Heterologous protein expression cassettes contained the prfA-dependent hly promoter, which drives the transcription of the gene encoding Listeriolysin O (LLO), and is activated within the microenvironment of the infected cell. Nucleotides 205586-206000 (414 bps) were amplified by PCR from *Listeria monocytogenes*, strain DP-L4056, using the primer pair shown below. The region amplified includes the hly promoter and also the first 28 amino acids of LLO, comprising the secA1 signal peptide (see above) and PEST domain. The expected sequence of this region for *Listeria monocytogenes*, strain EGD can be found in GenBank (Accession number: gi|16802048[ref|NC_003210.1|16802048]). The primers used in the PCR reaction are as follows:

[0443] Primer Pair:

Forward (KpnI-LLO nts. 1257-1276):
5'-CTCTGGTACCTCCTTTGATTAGTATATTC (SEQ ID NO:74)

Reverse (Bam HI-LLO nts.):
5'-CTCTGGATCCATCCGCGTGTTCCTTTTCG (SEQ ID NO:84)

[0444] (Restriction endonuclease recognition sites are underlined.)

[0445] The 422 bp PCR amplicon was cloned into the plasmid vector pCR-XL-TOPO (Invitrogen, Carlsbad, Calif.), according to the manufacturer's specifications. The nucleotide sequences of *Listeria*-specific bases in the pCR-XL-TOPO-hly promoter plasmid clone was determined. *Listeria monocytogenes* strain DP-L4056 contained eight nucleotide base changes flanking the prfA box in the hly promoter, as compared to the EGD strain. The hly promoter alignment for the *Listeria monocytogenes* DP-L4056 and EGD strains is shown in FIG. 1 below.

[0446] The 422 bp DNA corresponding to the hly promoter and secA1 LLO signal peptide were liberated from the pCR-XL-TOPO-hly promoter plasmid clone by digestion with Kpn I and Bam HI, and cloned into the pPL2 plasmid vector (Lauer et. al. 2002 J. Bact.), according to conventional methods well-known to those skilled in the art. This plasmid is known as pPL2-hlyP (native).

[0447] C. Shine-Dalgarno Sequence

[0448] At the 3' end of the promoter is contained a poly-purine Shine-Dalgarno sequence, the element required for engagement of the 30S ribosomal subunit (via 16S rRNA) to the heterologous gene RNA transcript and initiation of translation. The Shine-Dalgarno sequence has typically the following consensus sequence: 5'-NAGGAGGU-N₅₋₁₀-AUG (start codon)-3' (SEQ ID NO:85). There are variations of the poly-purine Shine-Dalgarno sequence. Notably, the *Listeria* hly gene that encodes listeriolysin O (LLO) has the following Shine-Dalgarno sequence: AAG-GAGAGTGAAACCCATG (SEQ ID NO:70) (Shine-Dalgarno sequence is underlined, and the translation start codon is bolded).

Example 4

Polynucleotides Encoding a Fusion Protein Comprising a secA1 Signal Peptide (LLO) and Human EphA2

[0449] The sequence of an expression cassette encoding the full-length human EphA2 antigen fused to a secA1 signal peptide (LLO signal peptide), plus the LLO PEST sequence, is shown in FIG. 2. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 3.

Example 5

Codon-Optimization of the Extracellular Domain of Human EphA2 (EX2)

[0450] The sequence encoding the extracellular domain of human EphA2 (amino acids 25-526) has been codon-optimized for expression in *Listeria monocytogenes*. The native

nucleotide sequence encoding the extracellular domain of human EphA2 is shown in FIG. 4. The nucleotide sequence for optimal codon usage in *Listeria* is shown in FIG. 5. The amino acid sequence of the extracellular domain of human EphA2 is shown in FIG. 6.

Example 6

Polynucleotides Encoding an Fusion Proteins
Comprising a secA1 Signal Peptide (LLO) and the
Extracellular Domain of huEphA2 (EX2)

[0451] A. Polynucleotide Without Codon-Optimization

[0452] The sequence of a polynucleotide encoding the extracellular domain of human EphA2 antigen fused to a secA1 signal peptide (LLO signal peptide), plus the LLO PEST sequence, is shown in FIG. 7. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 8.

[0453] B. Expression Cassette With Codon-Optimized Extracellular Domain of Human EphA2

[0454] The sequence of an expression cassette encoding the extracellular domain of human EphA2 antigen fused to a secA1 signal peptide (LLO signal peptide), plus the LLO PEST sequence, in which the sequence encoding the extracellular domain of EphA2 is codon-optimized for expression in *Listeria monocytogenes*, is shown in FIG. 9. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 10.

[0455] C. Expression Cassette With Codon-Optimized secA1 Signal Peptide and Codon-Optimized Extracellular Domain of Human EphA2

[0456] The sequence of an expression cassette encoding the extracellular domain of human EphA2 antigen fused to a secA1 signal peptide (LLO signal peptide), plus the LLO PEST sequence, where the sequences encoding the extracellular domain of EphA2, signal peptide and PEST sequence are all codon-optimized for expression in *Listeria monocytogenes*, is shown in FIG. 11. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 12.

Example 7

Codon-Optimized Expression Cassette Encoding a
Fusion Protein Comprising a Tat Signal Peptide (*B. subtilis* phoD) and Extracellular Domain of
huEphA2 (EX2)

[0457] The sequence of an expression cassette encoding the extracellular domain of EphA2 antigen fused to a Tat signal peptide (*B. subtilis* phoD) where the sequences encoding the extracellular domain of EphA2 and the signal peptide are all codon-optimized for expression in *Listeria monocytogenes*, is shown in FIG. 13. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 14.

Example 8

Codon-Optimization of the Intracellular Domain of
Human EphA2 (CO)

[0458] The sequence encoding the intracellular domain of human EphA2 (amino acids 558-975) has been codon-

optimized for expression in *Listeria monocytogenes*. The native nucleotide sequence encoding the extracellular domain of human EphA2 is shown in FIG. 15. The nucleotide sequence for optimal codon usage in *Listeria* is shown in FIG. 16. The amino acid sequence of the extracellular domain of human EphA2 is shown in FIG. 17.

Example 9

Polynucleotides Encoding Fusion Proteins
Comprising a secA1 Signal Peptide (LLO) and
Intracellular Domain of huEphA2 (CO)

[0459] A. Polynucleotide Without Codon-Optimization

[0460] The sequence of a polynucleotide encoding the intracellular domain of human EphA2 antigen fused to a secA1 signal peptide (LLO), plus the LLO PEST sequence, is shown in FIG. 18. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 19.

[0461] B. Expression Cassette With Codon-Optimized Intracellular Domain of Human EphA2

[0462] The sequence of an expression cassette encoding the intracellular domain of huEphA2 antigen fused to a secA1 signal peptide (LLO signal peptide), plus the LLO PEST sequence, in which the sequence encoding the intracellular domain of EphA2 is codon-optimized for expression in *Listeria monocytogenes*, is shown in FIG. 20. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 21.

[0463] C. Expression Cassette With Codon-Optimized secA1 Signal Peptide and Codon-Optimized Intracellular Domain of Human EphA2

[0464] The sequence of an expression cassette encoding the intracellular domain of EphA2 antigen fused to a secA1 signal peptide (LLO signal peptide), plus the LLO PEST sequence, where the sequences encoding the intracellular domain of EphA2, signal peptide and PEST sequence are all codon-optimized for expression in *Listeria monocytogenes*, is shown in FIG. 22. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 23.

Example 10

Codon-Optimized Expression Cassette Encoding a
Fusion Protein Comprising *B. subtilis* phoD Signal
Peptide and Intracellular Domain of huEphA2 (CO)

[0465] The sequence of an expression cassette encoding the intracellular domain of EphA2 antigen fused to a Tat signal peptide (*B. subtilis* phoD) where the sequences encoding the intracellular domain of EphA2 and the signal peptide are all codon-optimized for expression in *Listeria monocytogenes*, is shown in FIG. 24. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 25.

Example 11

Codon-Optimized Expression Cassette Encoding a
Fusion Protein Comprising LLO Signal Peptide and
NY-ESO-1

[0466] An expression cassette was designed for expression of the human testis cancer antigen NY-ESO-1 (Genbank

Accession No. NM_001327) in *Listeria monocytogenes*. The sequence of the expression cassette encoding the NY-ESO-1 fused to a secA1 signal peptide (LLO), plus the LLO PEST sequence, is shown in FIG. 26. The sequences coding for the antigen as well as the signal peptide in the expression cassette were codon-optimized for expression in *Listeria monocytogenes*. The amino acid sequence of the fusion protein encoded by this expression cassette is shown in FIG. 27.

Example 12

Codon-Optimized Expression Cassette for Encoding Antigens Fused to a Non-Listerial secA1 Signal Peptide (*L. Lactis* usp45)

[0467] An expression cassette was designed for expression of heterologous antigens in *Listeria monocytogenes* using a non-Listerial secA1 signal peptide. The amino acid sequence of the usp45 signal peptide from *Lactococcus lactis* (Steidler et al., *Nature Biotechnology*, 21:785-9 (2003)), its native coding sequence, and the coding sequence optimized for expression in *Listeria monocytogenes* is shown below.

Amino acid sequence: (SEQ ID NO:46)
 MKKKIISAILMSTVILSAAAPLSGVYA'DT
 Signal peptidase recognition site: VYA-DT (SEQ ID NO:55)
 Native nucleotide sequence: (SEQ ID NO:86)
 5' ATGAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACCTT
 TCTGCTGCAGCCCGTTGTCTAGGTGTTTACGCTGACACA3'
 Codons optimized for expression in *Listeria monocytogenes*: (SEQ ID NO:87)
 5' ATGAAAAAAGAAATTATTAGTGCAATTTTAATGAGTACAGTTATTTTA
 AGTGACAGCAGCACCATTAAAGTGGTGTGTTATGCAGATACA3'

[0468] The sequence of a partial expression cassette comprising the hly promoter from *Listeria monocytogenes* operably linked to the codon-optimized sequence encoding the Usp45 signal peptide is shown in FIG. 28. This sequence can be combined with either a codon-optimized or non-codon-optimized antigen sequence for expression of a fusion protein comprising the Usp45 signal peptide and the desired antigen.

Example 13

Codon-Optimized Expression Cassette and Vector for Encoding Antigens Fused to a secA2 Signal Peptide (p60)

[0469] A. Design of Codon-Optimized Expression Cassette

[0470] An expression cassette was designed for expression of heterologous antigens in *Listeria monocytogenes* using the secA2 secretion pathway. The amino acid sequence of the p60 signal peptide from *Listeria monocytogenes*, its native coding sequence, and the coding sequence optimized for expression in *Listeria monocytogenes* is shown below.

Amino acid sequence: (SEQ ID NO:48)
 MNMKKATIAATAGIAVTAEAAPTIASA'ST
 Signal peptidase recognition site: ASA-ST (SEQ ID NO:57)
 Native nucleotide sequence: (SEQ ID NO:90)
 5' ATGAATATGAAAAAGCAACTATCGCGGCTACAGCTGGGATTGCGGT
 AACAGCATTTGCTGCGCCAACAATCGCATCCGCAAGCACAJ'
 Codons optimized for expression in *Listeria monocytogenes*: (SEQ ID NO:91)
 5' ATGAATATGAAAAAGCAACAATTGCAGCAACAGCAGTATTGTCAGT
 TACAGCATTTGAGCAGCAACAATTGCAAGTCAAGTACA3'

[0471] The sequence of a partial expression cassette comprising the hly promoter from *Listeria monocytogenes* operably linked to the native sequence encoding the p60 signal peptide is shown in FIG. 29. The sequence of a partial expression cassette comprising the hly promoter from *Listeria monocytogenes* operably linked to the codon-optimized sequence encoding the p60 signal peptide is shown in FIG. 30.

[0472] B. Construction of pPL2-hlypro_p60.

[0473] An expression cassette can also be constructed in which the antigen-encoding sequence is inserted in frame in one or more sites within the coding sequence of the p60 gene. A description of the construction of a partial expression cassette useful for inserting antigen sequences in frame within the p60 sequence is described below. This partial expression cassette contains an hly promoter.

[0474] Individual primary PCR reactions using Pfx or Vent polymerase are performed using the following primers and pPL2-hlyP-OVA (identical to pPL2/LLO-OVA described above in Example 2A) as a first template:

pPL2-5F: (SEQ ID NO:92)
 5' -GACGTCAATACGACTCACTATAG
 p60-hlyP-237R: (SEQ ID NO:93)
 5' -CTTTTTCATATTCATGGGTTTCACTCTCTCTCTAC

[0475] The size of the resulting amplicon is 285 bps.

[0476] Individual primary PCR reactions using Pfx or Vent polymerase are also performed using the following primers and pCR-TOPO-p60 as a second template. (The vector pCR-TOPO-p60 is made from a pCR-TOPO vector obtained from Invitrogen, Carlsbad, Calif. in which the genomic p60 sequence from *Listeria monocytogenes* has been inserted. Any other of the many alternative sources of the p60 coding sequence that are available could be used as a template instead.) The primers used in this PCR reaction are as follows:

hlyP-p60-1 F: (SEQ ID NO:88)

-continued

5'-AAGGAGAGTGAAACCCATGAATATGAAAAAGCAAC

PCR-TOPO-2283R:

(SEQ ID NO:89)

5'-GTGTGATGGATATCTGCAGAATTC

[0477] The size of the resulting amplicon is 1510 bps. The PCR reactions are then cleaned with S6 columns (Bio-Rad Laboratories, Hercules, Calif.).

[0478] A secondary PCR reaction is then performed, using approximately 5 μ l of each primary PCR reaction as template. The secondary PCR reaction uses the following primers: KpnI-LLO 1257F (primer used previously): 5'-CTCTG-GTACCTCCTTTGATTAGTATATTC (SEQ ID NO:74) and pCR-TOPO-2258R: 5'-CCCTTGGGGATCCTTAAT-TATACG (SEQ ID NO:83). The size of the resulting amplicon is 1715 bps. The expected amplicon sizes in all PCR reactions are verified by agarose gel analysis. The secondary PCR reaction is cleaned, digested with BamHI, cleaned again, and digested with KpnI. The hlyP-p60 gene fragment (KpnI-BamHI) (FIG. 30) is then ligated between the BamHI and KpnI sites of both pPL2 and modified pAM401 (pAM401-MCS; FIG. 32) plasmids.

[0479] The construction of pPL2-p60 plasmid is then confirmed with BamH/KpnI (1697, 6024 bps) and HindIII (210, 424, 3460, 3634 bps) digests. The PstI site in pPL2-p60 plasmid is also confirmed as unique. (Also, KpnI/PstI digest will yield fragments of 736 and 6985 bps.) The construction of the pAM401-p60 plasmid (KpnI/PstI, and KpnI/BamHI fragments from p60 region is the same as that for the pPL2 construct.

[0480] Large prep isolations of each plasmid are then prepared using methods known to those of ordinary skill in the art.

[0481] The desired antigen-encoding sequences can then be inserted within the p60 sequence and in the same translational frame as the p60 sequence using techniques well known to those of ordinary skill in the art. Typically, the insertion or insertions should leave the N-terminal signal peptide sequence of p60 intact. The C-terminal autolysin sequence of p60 should also be left intact.

Example 14

Codon-Optimization of Human Mesothelin-Encoding Sequences for Expression in *Listeria monocytogenes*

[0482] A codon-optimized polynucleotide sequence encoding human mesothelin, a cancer antigen, is shown in FIG. 33. The sequence shown in FIG. 32 has been codon-optimized for expression in *Listeria monocytogenes*. The polypeptide sequence encoded by the sequence in FIG. 33 is shown in FIG. 34.

Example 15

Codon-Optimization of Murine Mesothelin-Encoding Sequences for Expression in *Listeria monocytogenes*

[0483] A codon-optimized polynucleotide sequence encoding human mesothelin, a cancer antigen, is shown in

FIG. 35. The sequence shown in FIG. 35 has been codon-optimized for expression in *Listeria monocytogenes*. The polypeptide sequence encoded by the sequence in FIG. 35 is shown in FIG. 36.

Example 16

Integration of an Expression Cassette into the *Listeria* Chromosome via Allelic Exchange

[0484] As one possible alternative to using an integration vector such as pPL2 to insert the heterologous gene expression cassette into the chromosome of *Listeria*, allelic exchange may be used.

[0485] Briefly, bacteria electroporated with the pKSV7-heterologous protein expression cassette plasmid are selected by plating on BHI agar media containing chloramphenicol (10 μ g/ml), and incubated at the permissive temperature of 30° C. Single cross-over integration into the bacterial chromosome is selected by passaging several individual colonies for multiple generations at the non-permissive temperature of 41° C. in media containing chloramphenicol. Finally, plasmid excision and curing (double cross-over) is achieved by passaging several individual colonies for multiple generations at the permissive temperature of 30° C. in BHI media not containing chloramphenicol. Verification of integration of the heterologous protein expression cassette into the bacteria chromosome is verified by PCR, utilizing a primer pair that amplifies a region defined from within the heterologous protein expression cassette to the bacterial chromosome targeting sequence not contained in the pKSV7 plasmid vector construct.

Example 17

Cloning and Insertion of EphA2 into pPL2 Vectors for Expression in Selected Recombinant *Listeria monocytogenes* Strains

[0486] The external (EX2) and cytoplasmic (CO) domains of EphA2 which flank the EphA2 transmembrane helix were cloned separately for insertion into various pPL2-signal peptide expression constructs. Genes corresponding to the native mammalian sequence or codon-optimized for expression in *Listeria monocytogenes* of EphA2 EX2 and CO domains were used. The optimal codons in *Listeria* (see Table 3, above) for each of the 20 amino acids were utilized for codon-optimized EphA2 EX2 and EphA2 CO. The codon-optimized EphA2 EX2 and CO domains were synthesized by extension of overlapping oligonucleotides, using techniques common to those skilled in the art. The expected sequence of all synthesized EphA2 constructs was verified by nucleotide sequencing.

[0487] The primary amino acid sequences, together with the native and codon-optimized nucleotide sequences for the EX2 and CO domains of EphA2 are shown in FIGS. 4-6 (EX2 sequences) and FIGS. 15-17 (CO domain sequences)

[0488] Additionally, FLAG (Stratagene, La Jolla, Calif.) and myc epitope tags were inserted, respectively, in-frame at the amino and carboxy termini of synthesized EphA2 EX2 and CO genes for detection of expressed and secreted EphA2 by Western blot analysis using antibodies specific for the FLAG or proteins. Thus, the expressed protein had the following ordered elements: NH₂-Signal Peptide-FLAG-

EphA2-myc-CO₂. Shown below are the FLAG and myc epitope tag amino acid and codon-optimized nucleotide sequences:

FLAG:
 5'-GATTATAAAGATGATGATGATAAA (SEQ ID NO:96)
 NH₂-DYKDDDDK-CO₂ (SEQ ID NO:97)
 Myc:
 5'-GAACAAAAATTAATTAGTGAAGAAGATTTA (SEQ ID NO:98)
 NH₂-EQKLISEEDL-CO₂ (SEQ ID NO:99)

Example 18

Detection of Synthesized and Secreted Heterologous Proteins by Western Blot Analysis

[0489] Synthesis of EphA2 protein and secretion from various selected recombinant *Listeria*-EphA2 strains was determined by Western blot analysis of trichloroacetic acid (TCA) precipitated bacterial culture fluids. Briefly, mid-log phase cultures of *Listeria* grown in BHI media were collected in a 50 mL conical centrifuge tube, the bacteria were pelleted, and ice-cold TCA was added to a final [6%] concentration to the bacterial culture supernatant and incubated on ice minimally for 90 min or overnight. The TCA-precipitated proteins were collected by centrifugation at 2400×g for 20 min at 4° C. The pellet was then resuspended in 300-600 µl volume of TE, pH 8.0 containing 15 µg/ml phenol red. Sample dissolution was facilitated by vortexing. Sample pH was adjusted by NH₄OH addition if necessary until color was pink. All samples were prepared for electrophoresis by addition of 100 µl of 4×SDS loading buffer and incubating for 10 min. at 90° C. The samples were then centrifuged from 5 min at 14,000 rpm in a micro-centrifuge, and the supernatants collected and stored at -20° C. For Western blot analysis, 20 µl of prepared fractions (the equivalent of culture fluids from 1-4×10⁹ bacteria), were loaded on the 4-12% SDS-PAGE gel, electrophoresed, and the proteins were transferred to PDDF membrane, according to common methods used by those skilled in the art. Transferred membranes were prepared for incubation with antibody, by incubating in 5% dry milk in PBS for 2 hr. at room temperature with agitation. Antibodies were used under the following dilutions in PBST buffer (0.1% Tween 20 in PBS): (1) Rabbit anti-Myc polyclonal antibody (ICL laboratories, Newberg, Oreg.) at 1:10,000; (2) murine anti-FLAG monoclonal antibody (Stratagene, La Jolla, Calif.) at 1:2,000; and, (3) Rabbit anti-EphA2 (carboxy terminus-specific) polyclonal antibody (sc-924, Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). Specific binding of antibody to protein targets was evaluated by secondary incubation with goat anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase and detection with the ECL chemiluminescence assay kit (Amersham), and exposure to film.

Example 19

Secretion of EphA2 Protein by Recombinant *Listeria* Encoding Various Forms of EphA2

[0490] A. *Listeria*: [strains DP-L4029 (actA) or DP-L4017 (LLO L461T)]

[0491] Expression Cassette Construct: LLOss-PEST-CO-EphA2

[0492] The native sequence of the EphA2 CO domain was genetically fused to the native secA1 LLO sequence, and the heterologous antigen expression cassette under control of the *Listeria* hly promoter was inserted into the pPL2 plasmid between the Kpn I and Sac I sites as described above. The pPL2-EphA2 plasmid constructs were introduced by conjugation into the *Listeria* strains DP-L4029 (actA⁻) and DP-L4017 (L461T LLO) as described above. FIG. 37 shows the results of a Western blot analysis of TCA-precipitated bacterial culture fluids of 4029-EphA2 CO and 4017-EphA2 CO. This analysis demonstrated that recombinant *Listeria* engineered to contain a heterologous protein expression cassette comprised of native sequences corresponding to the secA1 and EphA2 CO fusion protein secreted multiple EphA2-specific fragments that were lower than the 52 kDa expected molecular weight, demonstrating the need for modification of the expression cassette.

[0493] B. *Listeria*: [DP-L4029 (actA⁻)]

[0494] Expression Cassette Constructs:

[0495] 1. Native LLOss-PEST-FLA G-EX2_EphA2-myc-CodonOp

[0496] 2. (CodonOp) LLOss-PEST-(CodonOp)FLA G-EX2_EphA2-myc

[0497] The native secA1 LLO signal peptide sequence or secA1 LLO signal peptide sequence codon-optimized for expression in *Listeria* was fused genetically with the EphA2 EX2 domain sequence codon-optimized for expression in *Listeria*, and the heterologous antigen expression cassette under control of the *Listeria* hly promoter was inserted into the pPL2 plasmid between the Kpn I and Sac I sites as described above. The pPL2-EphA2 plasmid constructs were introduced by conjugation into the *Listeria* strain DP-L4029 (actA) as described above. FIG. 38 shows the results of a Western blot analysis of TCA-precipitated bacterial culture fluids of *Listeria* actA encoding either the native or codon-optimized secA1 LLO signal peptide fused with the codon-optimized EphA2 EX2 domain. This analysis demonstrated that the combination of utilizing sequence for both signal peptide and heterologous protein optimized for the preferred codon usage in *Listeria monocytogenes* resulted in expression of the expected full-length EphA2 EX2 domain protein. Expression of full-length EphA2 EX2 domain protein was poor with codon-optimization of the EphA2 coding sequence alone. The level of heterologous protein expression (fragmented or full-length) was highest when utilizing the *Listeria monocytogenes* LLO secA1 signal peptide, codon-optimized for expression in *Listeria monocytogenes*.

[0498] C. *Listeria*: [DP-L4029 (actA)]

[0499] Expression Cassette Constructs:

[0500] 3. Native LLOss-PEST-(CodonOp) FLAG-EphA2_CO-myc

[0501] 4. CodonOp LLOss-PEST-(CodonOp) FLAG-EphA2_CO-myc

[0502] 5. CodonOp PhoD-(CodonOp) FLAG-EphA2_CO-myc

[0503] The native secA1 LLO signal peptide sequence or the secA1 LLO signal peptide sequence codon-optimized for expression in *Listeria*, or, alternatively, the Tat signal peptide of the phoD gene from *Bacillus subtilis* codon-optimized for expression in *Listeria*, was fused genetically with the EphA2 CO domain sequence codon-optimized for expression in *Listeria*, and the heterologous antigen expression cassette under control of the *Listeria* hly promoter was inserted into the pAM401-MCS plasmid between the Kpn I and Sac I sites as described above. The pAM401-EphA2 plasmid constructs were introduced by electroporation into the *Listeria* strain DP-L4029 (actA) as described above. FIG. 39 shows the results of a Western blot analysis of TCA-precipitated bacterial culture fluids of *Listeria* actA encoding either the native or codon-optimized secA1 LLO signal peptide, or codon-optimized *Bacillus subtilis* phoD Tat signal peptide fused with the codon-optimized EphA2 CO domain. This analysis demonstrated once again that the combination of utilizing sequence for both signal peptide and heterologous protein optimized for the preferred codon usage in *Listeria monocytogenes* resulted in expression of the expected full-length EphA2 CO domain protein. Furthermore, expression and secretion of the expected full-length EphA2 CO domain protein resulted from recombinant *Listeria* encoding codon-optimized *Bacillus subtilis* phoD Tat signal peptide fused with the codon-optimized EphA2 CO domain. This result demonstrates the novel and unexpected finding that signal peptides from distinct bacterial species can be utilized to program the secretion of heterologous proteins from recombinant *Listeria*. Expression of full-length EphA2 CO domain protein was poor with codon-optimization of just the EphA2 sequence. The level of heterologous protein expression was highest when utilizing signal peptides codon-optimized for expression in *Listeria monocytogenes*.

[0504] D. Transfection of 293 Cells With pCDNA4 Plasmids Encoding Full-Length EphA2

[0505] Expression Cassette Constructs:

[0506] 6. pCDNA4-EphA2

[0507] The native full-length EphA2 gene was cloned into the eukaryotic CMV promoter-based expression plasmid pCDNA4 (Invitrogen, Carlsbad, Calif.). FIG. 40 shows the results of a Western blot analysis of lysates prepared from 293 cells transfected with the pCDNA4-EphA2 plasmid, and demonstrates the abundant expression in mammalian cells of full-length EphA2 protein.

Example 20

Therapeutic Efficacy in Balb/C Mice Bearing CT26 Tumors Encoding Human EphA2 Immunized With Recombinant *Listeria* Encoding Codon-Optimized EphA2

[0508] The following data presented in FIGS. 41-44 demonstrated the following:

[0509] Immunization of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors with recombinant *Listeria* encod-

ing OVA.AH1 (MMTV gp70 immunodominant epitope) or OVA.AH1-A5 (MMTV gp70 immunodominant epitope, with heteroclitic change for enhanced T-cell receptor binding) confers long-term survival (FIG. 41).

[0510] The EphA2 CO domain is strongly immunogenic, and a significant long term increase in survival of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors was observed when immunized with recombinant *Listeria* encoding codon-optimized or native EphA2 CO domain sequence (FIG. 43).

[0511] The EphA2 EX2 domain is poorly immunogenic, and increased survival of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors was observed only when immunized with recombinant *Listeria* encoding codon-optimized secA1 signal peptide fused with the codon-optimized EphA2 EX2 domain sequence. Therapeutic efficacy was not observed in mice when immunized with recombinant *Listeria* encoding native secA1 signal peptide fused with the codon-optimized EphA2 EX2 domain sequence (FIG. 42). The desirability of using both codon-optimized secA1 signal peptide and EphA2 EX2 domain sequences was supported by statistically significant therapeutic anti-tumor efficacy, as shown in Table 4, below.

TABLE 4

Comparison by log-rank test of survival curves shown in FIG. 42.

Experimental Group	Median Survival (Days)	Significance versus HBSS cohort (p value)	Significance versus actA-native secA1/EphA2 EX2 cohort (p value)
HBSS	19	—	—
actA	20	NS	NS
actA-native secA1-EphA2 EX2 (native)	19	NS	—
actA-native secA1-EphA2 EX2 (CodOp)	24	0.0035	NS
actA-CodOp secA1-EphA2 EX2 (CodOp)	37	0.0035	0.0162
actA-native secA1-EphA2 CO (CodOp)	>99	0.0035	0.0015

[0512] Significantly, even though pCDNA4-EphA2 plasmid transfected 293 cells yielded very high levels of protein expression, immunization of Balb/C mice bearing CT26.24 (huEphA2+) lung tumors with the pCDNA4-EphA2 plasmid did not result in any observance of therapeutic anti-tumor efficacy (FIG. 44).

[0513] For therapeutic in vivo tumor studies, female Balb/C mice were implanted IV with 5×10^5 CT26 cells stably expressing EphA2. Three days later, mice were randomized and vaccinated IV with various recombinant *Listeria* strains encoding EphA2. In some cases (noted in figures) mice were vaccinated with 100 μ g of pCDNA4 plasmid or pCDNA4-EphA2 plasmid in the tibialis anterior muscle. As a positive control, mice were vaccinated IV with recombinant *Listeria* strains encoding OVA.AH1 or OVA.AH1-A5 protein chimeras. Mice were vaccinated on days 3 and 14 following tumor cell implantation. Mice injected with Hanks Balanced Salt Solution (HBSS) buffer or unmodified *Listeria* served as negative controls. All experimental cohorts contained 5 mice. For survival studies

mice were sacrificed when they started to show any signs of stress or labored breathing.

Example 21

Assessment of Antigen-Specific Immune Responses After Vaccination

[0514] The vaccines of the present invention can be assessed using a variety of *in vitro* and *in vivo* methods. Some assays involve the analysis of antigen-specific T cells from the spleens of mice that have been vaccinated. Provided in this example are non-limiting examples of methods of assessing *in vitro* and *in vivo* immune responses. The antigens recited in these exemplary descriptions of assays are model antigens, not necessarily antigens produced using the recombinant nucleic acid molecules, expression cassettes, and/or expression vectors described herein. One of ordinary skill in the art will readily recognize that the assays described in this example can readily be applied for use in assessing the *in vitro* or *in vivo* immune responses of bacteria comprising the recombinant nucleic acid molecules, expression cassettes, and/or expression vectors described herein.

[0515] For example C57Bl/6 or Balb/c are vaccinated by intravenous injection of 0.1 LD₅₀ of a *Listeria* strain expressing OVA (or other appropriate antigen). Seven days after the vaccination, the spleen cells of the mice are harvested (typically 3 mice per group) by placing the spleens into ice cooled RPMI 1640 medium and preparing a single cell suspension from this. As an alternative, the lymph nodes of the mice could be similarly harvested, prepared as a single cell suspension and substituted for the spleen cells in the assays described below. Typically, spleen cells are assessed for intravenous or intraperitoneal administration of the vaccine while spleen cells and cells from lymph nodes are assessed for intramuscular, subcutaneous or intradermal administration of the vaccine.

[0516] Unless otherwise noted, all antibodies used in these examples can be obtained from Pharmingen, San Diego, Calif.

[0517] ELISPOT Assay: Using a *Listeria* strain having an OVA antigen as an example, the quantitative frequency of antigen-specific T cells generated upon immunization in a mouse model is assessed using an ELISPOT assay. The antigen-specific T cells evaluated are OVA specific CD8+ or LLO specific CD8+ or CD4+ T cells. This OVA antigen model assesses the immune response to a heterologous tumor antigen inserted into the vaccine and could be substituted with any antigen of interest. The LLO antigen is specific to *Listeria*. The specific T cells are assessed by detection of cytokine release (e.g. IFN- γ) upon recognition of the specific antigen. PVDF-based 96 well plates (BD Biosciences, San Jose, Calif.) are coated overnight at 4° C. with an anti-murine IFN- γ monoclonal antibody (mAb R4; 5 μ g/ml). The plates are washed and blocked for 2 hours at room temperature with 200 μ L of complete RPMI. Spleen cells from vaccinated mice (or non vaccinated control mice) are added at 2×10^5 cells per well and incubated for 20 to 22 hours at 37° C. in the presence of various concentrations of peptides ranging from 0.01 to 10 μ M. The peptides used for OVA and LLO are either SL8, an MHC class I epitope for OVA, LLO₁₉₀ (NEKYAQAYPNVS (SEQ ID NO: 100)

Invitrogen) an MHC class II epitope for listeriolysin O (*Listeria* antigen), LLO₂₉₆ (VAYGRQVYL (SEQ ID NO:101) an MHC class I epitope for listeriolysin O, or LLO₉₁ (GYKDGNEYI (SEQ ID NO:102)), an MHC class I epitope for listeriolysin O. LLO₁₉₀ and LLO₂₉₆ are used in a C57Bl/6 model, while LLO₉₁ is used in a Balb/c model. After washing, the plates are incubated with secondary biotinylated antibodies specific for IFN- γ (XMG1.2) diluted in PBS to 0.5 μ g/ml. After incubation at room temperature for 2 hours, the plates are washed and incubated for 1 hour at 37° C. with a 1 nm gold goat anti-biotin conjugate (GAB-1; 1:200 dilution; Ted Pella, Redding, Calif.) diluted in PBS containing 1% BSA. After thorough washing, the plates are incubated at room temperature for 2 to 10 minutes with substrate (Silver Enhancing Kit; 30 ml/well; Ted Pella) for spot development. The plates are then rinsed with distilled water to stop the substrate reaction. After the plates have been air-dried, spots in each well are counted using an automated ELISPOT plate reader (CTL, Cleveland, Ohio). The cytokine response is expressed as the number of IFN- γ spot-forming cells (SFCs) per 2×10^5 spleen cells for either the OVA specific T cells or the *Listeria* specific T cells

[0518] Intracellular Cytokine Staining Assay (ICS): In order to further assess the number of antigen-specific CD8+ or CD4+ T cells and correlate the results with those obtained from ELISPOT assays, ICS is performed and the cells evaluated by flow cytometry analysis. Spleen cells from vaccinated and control groups of mice are incubated with SL8 (stimulates OVA specific CD8+ cells) or LLO₁₉₀ (stimulates LLO specific CD4+ cells) for 5 hours in the presence of Brefeldin A (Pharmingen). The Brefeldin A inhibits secretion of the cytokines produced upon stimulation of the T cells. Spleen cells incubated with an irrelevant MHC class I peptide are used as controls. PMA (phorbol-12-myristate-13-acetate, Sigma) 20 ng/ml and ionomycin (Sigma) 2 μ g/ml stimulated spleen cells are used as a positive control for IFN- γ and TNF- α intracellular cytokine staining. For detection of cytoplasmic cytokine expression, cells are stained with FITC-anti-CD4 mAb (RM 4-5) and PerCP-anti-CD8 mAb (53-6.7), fixed and permeabilized with Cytofix/CytoPerm solution (Pharmingen), and stained with PE-conjugated anti-TNF- α mAb (MP6-XT22) and APC-conjugated anti-IFN- γ mAb (XMG1.2) for 30 minutes on ice. The percentage of cells expressing intracellular IFN- γ and/or TNF- α was determined by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, Calif.) and data analyzed using CELLQuest software (Becton Dickinson Immunocytometry System). As the fluorescent labels on the various antibodies can all be distinguished by the FACScalibur, the appropriate cells are identified by gating for those CD8+ and CD4+ that are stained with either or both of the anti-IFN- γ or anti-TNF- α .

[0519] Cytokine Expression of Stimulated Spleen Cells: The level of cytokine secretion by the spleen cells of mice can also be assessed for control and vaccinated C57Bl/6 mice. Spleen cells are stimulated for 24 hours with SL8 or LLO₁₉₀. Stimulation with irrelevant peptide HSV-gB2 (Invitrogen, SSIEFARL, SEQ ID NO:4) is used as a control. The supernatants of the stimulated cells are collected and the levels of T helper-1 and T helper 2 cytokines are determined using an ELISA assay (eBiosciences, CO) or a Cytometric Bead Array Kit (Pharmingen).

[0520] Assessment of Cytotoxic T cell Activity: The OVA specific CD8+ T cells can be further evaluated by assessing their cytotoxic activity, either in vitro or directly in C57Bl/6 mouse in vivo. The CD8+ T cells recognize and lyse their respective target cells in an antigen-specific manner. In vitro cytotoxicity is determined using a chromium release assay. Spleen cells of naïve and *Listeria*-OVA (internal) vaccinated mice are stimulated at a 10:1 ratio with either irradiated EG7.OVA cells (EL-4 tumor cell line transfected to express OVA, ATCC, Manassas, Va.) or with 100 nM SL8, in order to expand the OVA specific T cells in the spleen cell population. After 7 days of culture, the cytotoxic activity of the effector cells is determined in a standard 4-hour ⁵¹Cr-release assay using EG7.OVA or SL8 pulsed EL-4 cells (ATCC, Manassas, Va.) as target cells and EL-4 cells alone as negative control. The YAC-1 cell line (ATCC, Manassas, Va.) is used as targets to determine NK cell activity, in order to distinguish the activity due to T cells from that due to NK cells. The percentage of specific cytotoxicity is calculated as 100× (experimental release–spontaneous release)/(maximal release–spontaneous release). Spontaneous release is determined by incubation of target cells without effector cells. Maximal release is determined by lysing cells with 0.1% Triton X-100. Experiments are considered valid for analysis if spontaneous release is <20% of maximal release.

[0521] For the assessment of cytotoxic activity of OVA-specific CD8+ T cells in vivo, spleen cells from naïve C57Bl/6 mice are split into two equivalent aliquots. Each group is pulsed with a specific peptide, either target (SL8) or control (HSV-gB2), at 0.5 µg/ml for 90 minutes at 37° C. Cells are then washed 3 times in medium, and twice in PBS+0.1% BSA. Cells are resuspended at 1×10⁷ per ml in warm PBS+0.1% BSA (10 ml or less) for labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, Oreg.). To the target cell suspension, 1.25 µL of a 5 mM stock of CFSE is added and the sample mixed by vortexing. To the control cell suspension, a ten-fold dilution of the CFSE stock is added and the sample mixed by vortexing. The cells are incubated at 37° C. for 10 minutes. Staining is stopped by addition of a large volume (>40 ml) of ice-cold PBS. The cells are washed twice at room temperature with PBS, then resuspended and counted. Each cell suspension is diluted to 50×10⁶ per ml, and 100 µL of each population is mixed and injected via the tail vein of either naïve or vaccinated mice. After 12-24 hours, the spleens are harvested and a total of 5×10⁶ cells are analyzed by flow cytometry. The high (target) and low (control) fluorescent peaks are enumerated, and the ratio of the two is used to establish the percentage of target cell lysis. The in vivo cytotoxicity assay permits the assessment of lytic activity of antigen-specific T cells without the need of in vitro re-stimulation. Furthermore, this assay assesses the T cell function in their native environment.

Example 22

Human EphA2-Specific Immunity Induced by Vaccination of Balb/c Mice With *Listeria* Strains Expressing EphA2

[0522] Balb/c mice (n=3) were immunized with *Listeria* L461T expressing the intracellular domain of hEphA2 (*Listeria* hEphA2-ICD in FIG. 45) or an ΔactA (actA⁻) strain of *Listeria* expressing the extracellular domain of hEphA2

from a sequence codon-optimized for expression in *L. monocytogenes* (*Listeria* hEphA2-ECD in FIG. 45) two weeks apart. (The intracellular domain of hEphA2 is alternatively referred to herein as hEphA2-ICD, hEphA2 ICD, EphA2 CO, or CO. The extracellular domain of hEphA2 is alternatively referred to herein as hEphA2-ECD, hEphA2 ECD, EphA2 EX2, or EX2.) Mice were euthanized, and spleens harvested and pooled 6 days after the last immunization. For the ELISPOT assay, the cells were re-stimulated in vitro with P815 cells expressing full-length hEphA2 or cell lysates prepared from these cells. The parental P815 cells or cell lysates served as a negative control. Cells were also stimulated with recombinant hEphA2 Fc fusion protein. IFN-gamma positive spot forming colonies (SFCs) were measured using a 96 well spot reader. As shown in FIG. 45, increased IFN-gamma SFCs were observed with spleen cells derived from mice vaccinated with *Listeria*-hEphA2. Both hEphA2 expressing cells or cell lysates stimulation resulted in an increase in IFN-gamma SFC which suggests an EphA2-specific CD8+ as well as CD4+ T cell response. Spleen cells from mice vaccinated with the parental *Listeria* control did not demonstrate an increase in IFN-gamma SFC.

Example 23

CD4+ and CD8+ T Cell Responses are Required for EphA2 Specific Anti-Tumor Efficacy

[0523] Balb/c mice (n=10) were inoculated i.v. with 2×10⁵ CT26-hEphA2 on day 0. CD4+ cells and CD8+ T-cells were depleted by injecting 200 µg anti-CD4 (ATCC hybridoma GK1.5) or anti-CD8 (ATCC hybridoma 2.4-3) on Days 1 and 3, which was confirmed by FACS analysis (data not shown). Mice were then immunized i.v. with 0.1 LD₅₀ *Listeria* L461T expressing hEphA2 ICD on Day 4 and monitored for survival.

[0524] As shown in FIG. 46, both CD4+ and CD8+ depleted groups failed to demonstrate the anti-tumor response seen in the non-T cell depleted animals. The data are summarized in Table 5 below:

TABLE 5

Vaccination Group	Median Survival (Days)	P vs. HBSS	# Survivors (Day 67)
HBSS	17	—	0
<i>Listeria</i> -hEphA2-ICD	>67	<0.0001	7
<i>Listeria</i> -hEphA2-ICD + anti-CD4	19	0.03	2
<i>Listeria</i> -hEphA2-ICD + anti-CD8	24	0.0002	0

[0525] The foregoing data indicate a requirement for both CD4+ and CD8+ T cells in optimal suppression of tumor growth.

Example 24

Deletion of *inlB* from *Listeria* by Allelic Exchange

[0526] Bacteria comprising the recombinant nucleic acid molecules and expression cassettes described herein are, in some embodiments, mutant *Listeria*. For instance, in some embodiments, the bacteria comprising the recombinant nucleic acid molecules and expression cassettes are *Listeria monocytogenes* strains in which the *actA* gene, the *inlB* gene, or both, have been deleted. One exemplary method for generating a deletion mutant in *Listeria* is described below.

[0527] Deletion of the internalin B gene (*inlB*) from *Listeria* DP-L4029 (or from other selected mutant strains or from wild-type *Listeria*) can be effected by allelic exchange, as described by Camilli et al., Mol. Microbiol. 8:143-147 (1993). Splice Overlap Extension (SOE) PCR can be used to prepare the construct used in the allelic exchange procedure. The source of the internalin B gene is the sequence listed as Genbank accession number AL591975 (*Listeria monocytogenes* strain EGD, complete genome, segment 3/12; *inlB* gene region: nts. 97008-98963), incorporated by reference herein in its entirety, and/or the sequence listed as Genbank accession number NC_003210 (*Listeria monocytogenes* strain EGD, complete genome, *inlB* gene region: nts. 457008-458963), incorporated by reference herein in its entirety.

[0528] In the primary PCR reactions, approximately 1000 bps of sequence upstream and downstream from the *Listeria inlB* gene 5' and 3' ends, respectively, are amplified using the following template and primers:

[0529] Template: DP-L4056 or DP-L4029 genomic DNA

[0530] Primer pair 1 (For amplification of region upstream from 5' end of *inlB*):

Lm-96031F: (SEQ ID NO:103)

5'-GTTAAGTTTCATGTGGACGGCAAAG
(T_m : 72° C.)

Lm-(3' *inlB*-R +) 97020R:

(SEQ ID NO:104)
5'-AGGTCTTTTCAGTTAACTATCTCTCTGATTCTAGTTAT
(T_m : 114° C.)

[0531] (The underlined sequence complementary to region downstream of *InlB* carboxy terminus.)

[0532] Amplicon Size (bps): 1007

[0533] Primer pair 2 (For amplification of region downstream from 3' end of *inlB*):

Lm-(5' *inlB*-F +) 98911F: (SEQ ID NO:105)
5' CAAGGAGAGGATAGTTAACTGAAAAAGACCTAAAAAGAAGGC
(T_m : 118° C.)

[0534] (The underlined sequence complementary to region upstream of *InlB* amino terminus.)

Lm-99970R:
5'-TCCCCTGTTCTCTATAATTGTTAGCTC (SEQ ID NO:106)
(T_m : 74° C.)

Amplicon size (bps): 1074

[0535] In the secondary PCR reaction, the primary PCR amplicons are fused through SOE PCR, taking advantage of complementarity between reverse primer from pair 1 and the forward primer of pair 2. This results in precise deletion of *inlB* coding sequence: nts. 97021-98910=1889 bps. The following template and primers were utilized in the secondary PCR reaction:

[0536] Template: Cleaned primary PCR reactions

[0537] Primer pair:

Lm-96043F:
5'-GTGGACGGCAAAGAAACAACCAAAG (SEQ ID NO:107)
(T_m : 74° C.)

Lm-99964R:
5'-GTTCTCTATAATTGTTAGCTCATTTTTC (SEQ ID NO:108)
(T_m : 74° C.)

(Amplicon size (bps): 2033)

[0538] A protocol for completing the construction process is as follows:

[0539] The primary PCR reactions (3 temperature cycle) are performed using Vent DNA polymerase (NEB) and 10 μ l of a washed 30° C. *Listeria* DP-L4056 OR DP-L4029 overnight culture. The expected size of *Listeria* amplicons by 1% agarose gel (1007 bps and 1074 bps). The primary PCR reactions are gel purified and the DNA eluted with GeneClean (BIO 101).

[0540] A secondary PCR reaction is performed, utilizing approximately equal amounts of each primary reaction as template (ca. 5 μ l). The expected size of the *Listeria* amplicon from the secondary PCR reaction is verified by 1% agarose gel (2033 bps). Adenosine residue are added at the 3' ends of *Listeria* dl *inlB* amplicon with Taq polymerase.

[0541] The *Listeria* dl *inlB* amplicon is then inserted into a pCR2.1-TOPO vector. The pCR2.1-TOPO-dl *inlB* plasmid DNA is digested with XhoI and KpnI and the 2123 bp fragment is gel purified. The KpnI/XhoI 2123 bp fragment is inserted into a pKSV7 vector that has been prepared by digestion with KpnI and XhoI and treatment with CIAP (pKSV7-dl *inlB*). The fidelity of dl *inlB* sequence in pKSV7-dl *inlB* is then verified. The *inlB* gene is deleted from desired *Listeria* strains by allelic exchange with pKSV7-dl *inlB* plasmid.

Example 25

Codon-Optimized Signal Peptides for Construction of Recombinant *Listeria*

[0542] Some exemplary codon-optimized signal peptides that can be used in the expression cassettes in the recombinant *Listeria* are provided in Table 6, below.

TABLE 6

Exemplary signal peptides for construction of recombinant <i>Listeria</i>						
Secretion Pathway	Signal Peptide Amino Acid Sequence	Signal Site (') peptidase	peptidase Native Sequence	Sequence codon- expression in Lm	optimized for [Genus/species]	Gene
secA1	MKKIMLV	TEA'KD	ATGAAAAAAAAATAATG	ATGAAAAAAAAATTATGTT	hly (LLO)	
	FITLILVSL	(SEQ ID	CTAGTTTTTATTACAC	AGTTTTTATTACATTAAT	[<i>Listeria</i>	
	PIAQQTTEA	NO:54)	TTATATTAGTTAGTCT	TTTAGTTAGTTTACCAAT	<i>monocytogenes</i>]	
	KDASAFN		ACCAATTGCGCAACA	TGCACAACAACAGAAG		
	KENSISSM		AACTGAAGCAAAGGA	CAAAAGATGCAAGTGCA		
	APPASPPA		TGCATCTGCATTCAAT	TTTAATAAGAAAATAG		
	SPKTPIEK		AAAGAAAATTCAATT	TATTAGTAGTATGGCACC		
	KHAD		TCATCCATGGCACCA	ACCAGCAAGTCCACCAG		
	(SEQ ID		CCAGCATCTCGCCTG	CAAGTCCAAAAACACCA		
	NO: 109) ¹		CAAGTCCTAAGACGC	ATTGAAAAAACAATGC		
			CAATCGAAAAAGAAC	AGAT		
			ACGCGGAT (SEQ ID	(SEQ ID NO: 113)		
			NO:110)			
	MKKKIISA	VYA'DT	ATGAAAAAAGATT	ATGAAAAAATAATTAT	Usp45	
	ILMSTVILS	(SEQ ID	ATCTCAGCTATTTTAA	TAGTGCAATTTAATGAG	[<i>Lactococcus</i>	
	AAAPLSG	NO:55)	TGCTACAGTGATACT	TACAGTTATTTAAGTGC	<i>lactis</i>]	
	VYADT		TFCTGCTGGAGCCCCG	AGCAGCACCATTAAAGTG		
	(SEQ ID		TTGTTCAGGTGTTTACG	GTGTTTATGCAGATACA		
	NO:46)		CTGAGACA (SEQ ID	(SEQ ID NO:87)		
			NO:86)			
	MKKRKVL	IQA'EV	ATGAAAAACGAAAA	ATGAAAAACGTAAAGT	pag	
	IPLMALSTI	(SEQ ID	GTGTTAATACCATTAA	TTTAATCCATTAAATGGC	(Protective	
	LVSSTGNL	NO:56)	TGGCATTGTCTACGAT	ATTAAGTACAAATTTAGT	Antigen)	
	EVIQAEV		ATTAGTTTCAAGCAC	TAGTAGTACAGGTAATTT	[<i>Bacillus</i>	
	(SEQ ID		AGGTAATTTAGAGGT	AGAAGTTATTCAAGCAG	<i>anthracis</i>]	
	NO:47)		GATTCAAGCAGAAAGT	AAGTT (SEQ ID		
			T (SEQ ID NO:	NO: 114)		
			111)			
secA2	MNMKKAT	ASA'ST	ATGAATATGAAAAAA	ATGAATATGAAAAAGC	iap	
	IAATAGIA	(SEQ ID	GCAACTATCGCGGCT	AACAATTGCAGCAACAG	invasion-	
	VTAFAPPT	NO:57)	ACAGCTGGGATTGCG	CAGGTATTGCAGTTACAG	associated pro-	
	IASAST		GTAACAGCATTGCT	CATTTCGAGCACCAACA	tein	
	(SEQ ID		GCGCCAACAATCGCA	ATTGCAAGTGCAAGTAC	p60	
	NO:48)		TCCGCAAGCACT	A (SEQ ID NO:91)	[<i>Listeria</i>	
			(SEQ ID NO:90)		<i>monocytogenes</i>]	
Tat	MAYDSRF	VGA'F	ATGGCATACGACAGT	ATGGCATATGATAGTCGT	PhoD	
	DEWQKL	(SEQ ID	CGTTTTGATGAATGG	TTTGATGAATGGGTTCAA	alkaline	
	KESFQNN	NO:62)	GTACAGAAACTGAAA	AAATTAAGAAGAAAG	phosphatase	
	TFRRRFKI		GAGGAAAGCTTTCAA	TTTTCAAAATAATACATT	[<i>Bacillus</i>	
	QGAGKIA		AACAATACGTTTAC	TGATCGTCGTAATTTAT	<i>subtilis</i>]	
	GLSLGLTI		CGCCGCAAAATTTATC	TCAAGGTGCAGGTAAAA		
	AQSVGAF		AAGGAGCGGGGAAGA	TTGCAGGTTTAAGTTTAG		
	(SEQ ID		TTGCAGGACTTTCTCT	GTTTAAACAATTCACAAA		
	NO:53)		TGGATTAAACGATTGC	GTGTTGGTGCAATT		
			CCAGTCGGTTGGGGC	(SEQ ID NO:115)		
			CTTT (SEQ ID			
			NO:112)			

¹The sequence shown includes the PEST sequence from LLO.

Example 26

Codon-Optimized Expression Cassette Comprising
Bacillus anthracis Protective Antigen (PA) Signal
 Peptide

[0543] An expression cassette was designed for expression of heterologous antigens in *Listeria monocytogenes* using a non-*Listeria* secA1 signal peptide. The amino acid sequence of the Protective Antigen (PA) signal peptide from *Bacillus anthracis* (Ba) (GenBank accession number NC_007322), its native coding sequence, and the coding

sequence optimized for expression in *Listeria monocytogenes* are shown below.

Amino acid sequence:

MKKRKVL IPLMALSTILVSSTGNLEVIQAEV

(SEQ ID NO:47)

Signal peptidase recognition site:
 IQA'EV (SEQ ID NO:56)

-continued

Native nucleotide sequence: (SEQ ID NO:111)
 ATGAAAAACGAAAGTGTTAATACCATTAAATGGCATTGCTACGATATT
 AGTTTCAAGCACAGGTAATTTAGAGGTGATTCAAGCAGAAGTT

Codons optimized for expression in *Listeria monocytogenes*: (SEQ ID NO:114)
 ATGAAAAACGTAAGTTTAAATTCATTAAATGGCATTAAATGATCAATTT
 TAGTTAGTAGTACAGGTAATTTAGAAGTTATTCAAGCAGAAGTT

[0544] The sequence of a partial expression cassette comprising the hly promoter from *Listeria monocytogenes* operably linked to the codon-optimized sequence encoding the Ba PA signal peptide is shown in FIG. 47. This sequence can be combined with either a codon-optimized or non-codon-optimized antigen sequence for expression of a fusion protein comprising the *Bacillus anthracis* PA signal peptide and the desired antigen.

Example 27

Expression and Secretion of Antigens from Recombinant *Listeria* Comprising Codon-Optimized Expression Cassettes

[0545] Codon optimization of both signal peptide and tumor antigen provides efficient expression and secretion from recombinant *Listeria*: Codon-optimization of both signal peptide- and heterologous protein-encoding genetic elements provides optimal secretion from recombinant *Listeria*-based vaccines of human tumor antigens that contain hydrophobic domains. Efficient antigen secretion from cytosolic bacteria is required for efficient presentation via the MHC class I pathway and CD8+ T-cell priming, and is thus linked directly to the potency of *Listeria*-based vaccines. Secretion from recombinant *Listeria* of two malignant cell membrane-bound human tumor antigens, mesothelin and NY-ESO-1, which are immune targets related to pancreatic and ovarian cancer (mesothelin), and melanoma (NY-ESO-1), among other solid tumors, has been optimized through codon-optimization of the combination of both the antigen and signal peptide coding sequences.

[0546] A variety of expression cassettes were constructed comprising the hly promoter linked to either native or codon-optimized sequences encoding signal peptides related to secA1 or alternative secretion pathways including secA2 and Twin-Arg Translocation (Tat), fused in frame with a selected human tumor antigen—human NY-ESO-1 or human mesothelin. (See Examples 11-14 and 25, above, for the antigen sequences and/or signal sequences.) Western blot analysis of TCA-precipitated culture fluids of *Listeria* grown in BHI broth was used to assess the synthesis and secretion of the heterologous proteins from the recombinant *Listeria*. (Methods analogous to those described in Example 18, above, were used for the Western blot analyses.)

[0547] The results of these experiments are shown in FIG. 48A-C. Efficient expression and secretion of full-length tumor antigens from recombinant *Listeria* was observed when both signal peptide coding sequences, including when derived from *Listeria monocytogenes*, and operably linked foreign antigen coding sequences were optimized for codon

usage in *Listeria monocytogenes*. FIG. 48A shows the expression/secretion of human mesothelin by Δ actA *Listeria monocytogenes* with a construct comprising an LLO signal peptide fused with human mesothelin, using native codons for both LLO and mesothelin. By Western analysis of TCA-precipitated bacterial culture fluids, secretion of expected full-length mesothelin (62 kDa) was not observed with these constructs, and only secretion of several small fragments was observed (FIG. 48A).

[0548] FIG. 48B shows a Western blot analysis of the expression/secretion of human mesothelin by *Listeria monocytogenes* Δ actA comprising plasmids (pAM401) containing constructs encoding various signal peptides fused with human mesothelin. In each construct, the mesothelin coding sequence was codon-optimized for expression in *Listeria monocytogenes*. Where indicated, the signal peptide coding sequences used contained either the native sequence ("native") or were codon-optimized ("CodOp") for expression in *Listeria monocytogenes*. Secreted mesothelin was detected using an affinity-purified polyclonal anti-human/mouse antibody, prepared by injection of rabbits with selected peptides together with IFA.

[0549] Significantly, as shown in lanes 3-5, and 8-9 of FIG. 48B, secretion of full-length mesothelin (62 kDa) was observed only when both signal peptide and mesothelin coding sequences were codon-optimized for expression in *Listeria*. This observation significantly also included the *Listeria*-derived signal peptides from the bacterial LLO and p60 proteins, related to the secA1 and secA2 secretion pathways, respectively, both of which contain infrequently-used codons. (The LLO PEST sequence is also included with the LLO signal peptide and its coding sequence is also codon-optimized.) Efficient secretion of full-length mesothelin (62 kDa) was observed when the codon-optimized *Listeria* LLO signal peptide was linked with codon-optimized mesothelin (Lane 8, FIG. 48B), but NOT when the native coding sequence of the *Listeria* LLO signal peptide was used (Lane 7, FIG. 48B). Furthermore, secretion of full-length mesothelin (62 kDa) was observed when the codon-optimized *Listeria* p60 signal peptide was linked with codon-optimized mesothelin (Lane 3, FIG. 48B), but NOT when the native coding sequence of the *Listeria* p60 signal peptide was used (Lane 6, FIG. 48B). Finally, secretion of full-length mesothelin (62 kDa) was observed when codon-optimized optimized signal peptides from bacterial species different from *Listeria monocytogenes* were operably linked to codon-optimized mesothelin (FIG. 48B). The signal peptide from *Bacillus anthracis* protective antigen (Ba PA), or the signal peptide from *Lactococcus lactis* Usp45 protein (Ll Usp45) programmed the efficient secretion of full-length mesothelin (62 kDa) from the recombinant *Listeria* strains (FIG. 48B, lanes 4 and 5). The *Bacillus subtilis* phoD signal peptide (Bs phoD) also programmed the efficient secretion of full-length mesothelin from *Listeria* (FIG. 48B, lane 9). The bands with a molecular weight of about 62,000 correspond to mesothelin and the pairs of double bands probably correspond to non-cleaved plus cleaved mesothelin polypeptides (i.e., to partial cleavage).

[0550] FIG. 48C shows the expression/secretion of NY-ESO-1 from *Listeria monocytogenes* Δ actA Δ inlB with constructs comprising a sequence encoding LLO signal peptide which was fused with a sequence encoding human NY-ESO-

1, both of which were codon-optimized for expression in *Listeria*. Secreted NY-ESO-1 was detected using a NY-ESO-1 monoclonal antibody.

[0551] In this example, signal peptide and tumor antigen domains were synthesized to utilize the most preferred codon for each amino acid, as defined by frequency of occurrence per 1000 codons in coding sequences from the *Listeria* genome ([http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Listeria+monocytogenes+\[gbt\]](http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Listeria+monocytogenes+[gbt])). Signal peptides related to secA1, secA2, or twin-Arg translocation (Tat) secretion pathways from *Listeria* and other Gram-positive bacterial genera programmed the efficient secretion of human tumor antigens from recombinant *Listeria*. Surprisingly, the signal peptides from *Listeria* proteins LLO and p60 each contain rare codons (frequency of <10 per 1000 codons), and optimization of these sequences was required for efficient secretion of mesothelin and NY-ESO-1 from recombinant *Listeria* (FIG. 48B). Mesothelin secretion was also observed when linked to secA1 signal peptides from *B. anthracis* protective antigen (pagA) and *Lactococcus lactis* Usp45, and the Tat signal peptide from the phosphodiesterase/alkaline phosphatase D gene (PhoD) of *B. subtilis*.

[0552] Signal peptides from distinct secretion pathways were used to determine whether a particular pathway would be favored for optimal secretion of heterologous proteins. For example, the Tat pathway is utilized for secretion of proteins folded within the bacterium, and the *B. subtilis* phoD protein is secreted via this mechanism. It had originally been hypothesized that secretion of tumor antigens containing significant hydrophobic domains, such as NY-ESO-1, might be facilitated by folding prior to transport. However, these results indicated that codon-optimization of both the signal peptide and tumor antigen encoding sequences, and not secretion pathway, is the primary requirement for efficient secretion of mammalian proteins.

[0553] Importantly, the phenotype of recombinant vaccines utilizing any pathway for tumor antigen secretion was not significantly affected, as compared to the parental *Listeria* Δ actA/ Δ inlB strain. The median lethality (LD₅₀) of *Listeria* Δ actA/ Δ inlB is 1×10^8 cfu in C57BL/6 mice. Stable single copy site-specific incorporation of tumor antigen expression cassettes into an innocuous site on the chromosome of *Listeria* Δ actA/ Δ inlB, was accomplished using the pPL2 integration vector. The LD₅₀ of tumor antigen encoding *Listeria* Δ actA/ Δ inlB was within 5-fold of *Listeria* Δ actA/ Δ inlB.

Example 28

Construction of Bicistronic hEphA2 Expression Vectors

[0554] As a non-limiting example, construction of an antigen expression cassette, in which expression of the external (EX2) and internal (CO; kinase dead) domains of hEphA2 occurs from a bicistronic message, is given. Secretion of the EX2 and CO domains is accomplished by functional linkage of the Ba PA and Bs PhoD signal peptides with the EX2 and CO domains, respectively.

[0555] A codon-optimized human EphA2 kinase dead plasmid, known as phEphA2KD, is used in the construction of a bicistronic hEphA2 expression vector. (EphA2 is a

receptor tyrosine kinase, but the kinase activity is ablated by a mutation from K to M at the active site of the enzyme.) The coding sequences of phEphA2KD are shown in FIG. 49. The phEphA2KD sequence in FIG. 49 comprises the codon-optimized coding sequence for hEphA2 deleted of the transmembrane domain, and contains unique 5' and 3' Bam HI and Sac I restriction sites to facilitate construction of functional antigen expression cassettes. Mlu I recognition sequences are shown bolded in the sequence shown in FIG. 49.

[0556] A sub-fragment of the human EphA2 (trans-membrane domain deleted, kinase-dead) between the two Mlu I restriction enzyme recognition sequences is synthesized (by a gene synthesis method known in the art, e.g., by oligonucleotide synthesis, PCR, and/or Klenow fill-in, or the like). The actA-plcB intergenic region is inserted during the synthesis precisely at the junction between the EphA2 extracellular and intracellular domains, which are separated by the hydrophobic trans-membrane domain in the native protein. The sequence of the Mlu I sub-fragment of codon-optimized human EphA2 containing the actA-plcB intergenic region is shown in FIG. 50 (the intergenic region is shown in bold). Additionally, the codon-optimized Bs phoD signal peptide is placed at the 3' end of the actA-plcB intergenic sequence and is fused in-frame with the downstream EphA2 CO domain coding region.

[0557] The functional human EphA2 bicistronic cassette is assembled by substitution of the Mlu I fragment containing the actA-plcB intergenic region and Bs phoD signal peptide for the corresponding region in the trans-membrane domain deleted kinase dead human EphA2 sequence shown in FIG. 49. This resulting sequence contains unique Bam HI and Sac I restriction enzyme recognition sites at its 5' and 3' ends, respectively, to facilitate insertion and functional linkage to the hly promoter and initial signal peptide, for example Ba PA.

[0558] Thus, the seven ordered functional elements of the bicistronic human EphA2 antigen expression cassette are the following: hly promoter-Ba PA signal peptide-EX domain EphA2-termination codon-actA-plcB intergenic region (with Shine-Dalgarno sequence)-Bs PhoD signal peptide-CO domain EphA2-termination codon. All EphA2 and signal peptide coding sequences are preferably codon-optimized.

[0559] Recombinant *Listeria* strains that express and secrete the EphA2 EX and CO domains can be derived by methods illustrated in this application, utilizing the pAM401, pKSV7, or pPL1 and pPL2 integration vectors. Expression and secretion of the EphA2 proteins is detected by Western analysis of desired bacterial fractions, using methods described herein and/or known to those skilled in the art.

Example 29

Expression and Secretion of Antigens from Recombinant *Listeria* Comprising Antigen-Bacterial Protein Chimeras

[0560] In some embodiments of the invention, both the sequences encoding the signal peptide and its heterologous protein fusion partner are codon-optimized. In some embodiments, it is desirable to place the codon-optimized

heterologous protein sequence within a defined region of a protein, whose native form is secreted from *Listeria*. The heterologous protein sequence is functionally placed within a defined sequence of the selected secreted *Listeria* protein sequence such that a protein chimera is synthesized and secreted that corresponds to the combined molecular weights of the secreted proteins. Secretion of the heterologous protein can be facilitated by exploiting the machinery of the host *Listeria* bacterium that is required for optimal secretion of autologous bacterial proteins. Molecular chaperones facilitate secretion of selected bacterial proteins.

[0561] As a non-limiting example, protein chimeras between the *L. monocytogenes* protein p60 and the human tumor antigen, mesothelin, were generated. The protein chimeras were generated by precise placement of the human tumor antigen, mesothelin, into *L. monocytogenes* protein p60 at amino acid position 70 (although it is understood that any desired heterologous protein encoding sequence can be selected to generate a protein chimera). The protein chimera contained optimal codons for expression in *Listeria* in the p60 amino acids 1-70 and the entire mesothelin coding sequence. Furthermore, the p60-human mesothelin protein chimera was functionally linked to the *L. monocytogenes* hly promoter, incorporated into the pPL2 vector, which was used subsequently as described herein to generate recombinant *L. monocytogenes* strains expressing and secreting human mesothelin. The experimental methods used to construct a recombinant *Listeria* strain that optimally expresses and secretes a p60-human mesothelin protein chimera are described below.

[0562] In some embodiments, an important feature of protein chimeras between a selected *L. monocytogenes* gene and a selected heterologous protein sequence is appropriate functional placement of the selected heterologous protein sequence within the selected *L. monocytogenes* gene to retain optimal secretion of the protein chimera through interaction of the *L. monocytogenes* expressed protein with the bacterial chaperones and secretion apparatus, as well as to retain functional activity of the *L. monocytogenes* protein in the context of the protein chimera. In some embodiments, functional placement of a heterologous sequence within the *L. monocytogenes* secA2-dependent proteins NamA and p60 is desired to retain the peptidoglycan cell wall hydrolase activities of these said proteins. (See Lenz et. al. (2003) PNAS, 100:12432-12437, for instance, for descriptions of the SecA2-dependent NamA and p60 proteins.) In some embodiments, the functional placement of the heterologous protein coding sequence is desired between the signal sequence (SS) and the cell wall binding domains (LySM) and catalytic domains Lyz-2 (NamA) and p60-dom (p60) (Lenz et. al. (2003)).

[0563] In some embodiments, expression of antigens or heterologous proteins is functionally linked to a prfA-dependent promoter. As such, expression of the heterologous protein is induced within the microenvironment of the recombinant *Listeria* infected cell.

[0564] The first step in the construction of a p60-Mesothelin protein chimera involved the DNA synthesis of the prfA-dependent hly promoter linked functionally to a DNA sequence encoding the first 70 amino acids of p60, with codons for optimal secretion in *Listeria*. (In some embodiments, the codon usage can be modified further to avoid

regions of excessive RNA secondary structure, which may inhibit protein translation efficiency.) The DNA sub-fragment corresponding to the hly promoter-70 N-terminal p60 amino acids was synthesized. (This can generally be done by a gene synthesis method known in the art, e.g., by oligonucleotide synthesis, PCR, and/or Klenow fill-in, or the like.)

[0565] The sequence of the first 70 amino acids of p60 from *L. monocytogenes*, strain 10403S, is shown below:

(SEQ ID NO:116)
M N M K K A T I A A T A G I A V T A F A A P T I A
S A S T V V V E A G D T L W G I A Q S K G T T V D
A I K K A N N L T T D K I V P G Q K L Q

[0566] It can be appreciated to those skilled in the art that there exists multiple laboratory and field isolates of *L. monocytogenes* encoding genes, including p60, that may contain variability at both the nucleotide sequence and amino acid level, but are nevertheless essentially the same gene and protein. Furthermore, it can be appreciated by those skilled in the art that protein chimeras can be constructed utilizing genes from any laboratory or field isolate (including food-borne or clinical strain) of *L. monocytogenes*.

[0567] The synthesized DNA sequence corresponding to the hly promoter-70 N-terminal p60 amino acids is shown in FIG. 51. Furthermore, the codons encoding p60 amino acid residues 69 (L) and 70 (Q), were modified to contain a unique Pst I enzyme recognition sequence, to facilitate functional insertion of a heterologous sequence. Furthermore, the 5' end of the synthesized sub-fragment contains a unique KpnI enzyme recognition sequence.

[0568] The 447 bp KpnI and PstI digested sub-fragment fragment was ligated into the corresponding KpnI and PstI sites of the pPL2 vector, and treated by digestion with KpnI and PstI enzymes and digestion with calf intestinal alkaline phosphatase (CIAP). This plasmid is known as pPL2-hlyP-Np60 CodOp. Subsequently, the remainder of the native p60 gene was cloned into the pPL2-hlyP-Np60 CodOp plasmid, between the unique Pst I and BamHI sites. The remainder of the p60 gene was cloned by PCR, using a proof-reading containing thermostable polymerase, and the following primer pair:

Forward primer:
5'- CGC CTGCAGGTAATAATGAGGTTGCTG (SEQ ID NO:117)

Reverse primer:
5'-CGCGGATCCTTAATTATACGCGACCGAAG (SEQ ID NO:118)

[0569] The 1241 bp amplicon was digested with PstI and BamHI, and the purified 1235 bp was ligated into the pPL2-hlyP-Np60 CodOp plasmid, digested with PstI and BamHI, and treated with CIA-P. This plasmid contains the full *L. monocytogenes* p60 gene with optimal codons corresponding to amino acids 1-77, and native codons corresponding to amino acids 78-478, and is linked functional to the *L. monocytogenes* hly promoter. This plasmid is known as pPL2-hlyP-Np60 CodOp(1-77), and the sequence of the KpnI-BamHI sub-fragment that contains the hlyP linked

functionally to the p60 encoding sequence is shown in FIG. 52. The expected sequence of the pPL2-hlyP-Np60 CodOp(1-77) plasmid was confirmed by sequencing.

[0570] The next step in the construction was the functional insertion of a heterologous protein encoding sequence at the unique PstI site of plasmid as pPL2-hlyP-Np60 CodOp(1-77), which is between the N-terminal signal sequence and the first LysM cell wall binding domain of p60, thus retaining the normal biological function of the *L. monocytogenes* protein.

[0571] As a non-limiting example, human mesothelin that was codon-optimized for optimal expression in *L. monocytogenes* protein was inserted into the unique PstI site of plasmid as pPL2-hlyP-Np60 CodOp(1-77). Specifically, full-length mesothelin, or mesothelin that was deleted of the signal peptide and GPI linker domains (Mesothelin Δ SP/ Δ GPI) was cloned from the plasmid described in Example 27 that contains the full-length human mesothelin, containing optimal codons for expression in *L. monocytogenes*, using a thermostable polymerase with proof-reading activity, and the following primer pair:

[0572] 1. Full Length

Forward Primer (huMeso 3F):

5'-AAACTGCAGGCATTGCCAACTGCACGTCC (SEQ ID NO:119)

Reverse Primer (hMeso 1935R):

5'-AAACTGCAGAGCTAATGTACTGGCTAATAATAATGCTAAC (SEQ ID NO: 120)

[0573] 2. Δ Signal Peptide, Δ GPI Anchor

Forward Primer (huMeso 133F):

5'-CGCCTGCAGCGTACATTAGCAG GTGAACAGG (SEQ ID NO:121)

5'-CGCCTGCAGGCCTTGTAACCTAAAC CTAATGTATC (SEQ ID NO:122)

[0574] The PCR amplicons of 1932 bps (full-length mesothelin) and 1637 bps (Mesothelin Δ SP/ Δ GPI) were purified, digested with PstI, purified, and ligated into the unique PstI site of plasmid pPL2-hlyP-Np60 CodOp(1-77), treated by digestion with PstI, and treatment with CIAP. The consistent N-CO orientation of the p60 and mesothelin domains was confirmed by restriction endonuclease mapping. These plasmids are known as pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin and pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin Δ SP/ Δ GPI, and were introduced into selected *L. monocytogenes* strains (such as Δ actA Δ inlB double deletion mutants), as described throughout the examples contained herein.

[0575] The sequence of the KpnI-BamHI sub-fragment of plasmid pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin containing the hly promoter linked functionally to the p60-human Mesothelin protein chimera encoding gene is shown in FIG. 53.

[0576] The sequence of the KpnI-BamHI sub-fragment of plasmid pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin Δ SP/ Δ GPI containing the hly promoter linked functionally to the p60-human mesothelin Δ SP/ Δ GPI protein chimera encoding gene is shown in FIG. 54.

[0577] Western Analysis of Expression and Secretion of p60-Mesothelin Protein Chimeras:

[0578] As discussed throughout the examples, expression and secretion of a selected heterologous antigen results in potent priming of MHC class I-restricted CD8+ T cell responses. The expression and secretion of the protein chimeras into the media by recombinant *L. monocytogenes* Δ actA Δ inlB double deletion mutants containing tRNA-Arg chromosomal insertions of the pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin or pPL2-hlyP-Np60 CodOp(1-77)-Mesothelin Δ SP/ Δ GPI plasmids, generated by methods described herein, were tested by Western analysis by methods described in the Examples contained herein, using a mesothelin-specific polyclonal antibody.

[0579] The indicated engineered deletions in hMesothelin (Δ SP/ Δ GPI, also referred to herein as Δ SS/ Δ GPI, Δ SP/ Δ GPI, Δ SS/ Δ GPI, etc.) for the proteins shown in some of the lanes were as follows: The deleted signal sequence (Δ SP) corresponds to the N-terminal 34 amino acids of hMesothelin (for sequences of human mesothelin, see, e.g., FIG. 34 or GenBank Acc. No. BC009272). The deleted GPI (Δ GPI) domain corresponds to the C-terminal 42 amino acids, beginning with the amino acid residues Gly-Ile-Pro and ending with the amino acid residues Thr-Leu-Ala (see, e.g., FIG. 34).

[0580] The results of this analysis demonstrated that protein chimeras comprised of p60 with precise insertion of human mesothelin or human mesothelin Δ SP/ Δ GPI (inserted in frame at amino acid 70 of p60 between the N-terminal signal sequence and the first of two LysM cell wall binding domains) were efficiently expressed and secreted from the recombinant *L. monocytogenes*. See FIG. 55. (The Y-axis of FIG. 55 shows the molecular weight (in kDa) of proteins in the ladder run in the far left lane.) Specifically, lanes 1-4 in FIG. 55 demonstrate the expression and secretion of the expected protein chimeras containing human mesothelin or human mesothelin Δ SP/ Δ GPI. The increased efficiency of expression and secretion of human mesothelin Δ SP/ Δ GPI relative to the full-length mesothelin is evident in lanes 2 and 4. In the protein chimeras shown in lanes 3 and 4, the authentic N-terminal p60 amino acids were used. In the chimeras run in lanes 1 and 2 in the FIG. 55, the nucleotides encoding amino acids T and V at positions 29 and 64, respectively, were deleted. Lane 5 shows expression and secretion of *Bacillus anthracis* PA signal peptide fused to human Δ SP/ Δ GPI-mesothelin (where both the signal peptide and the mesothelin coding sequences were codon-optimized for expression in *L. monocytogenes*), and lane 6 shows the expression and secretion of LLO fused to full-length human mesothelin (where both the signal peptide and the mesothelin coding sequences were codon-optimized for expression in *L. monocytogenes*). Lane 8 shows protein expression by J293, a human cell line, while lane 7 shows protein expressed and secreted by J293 containing a plasmid encoding full-length human mesothelin ("J293/Full Length"). Lane 10 shows protein expression and secretion from *Listeria* which has been deleted of endogenous p60. The lower panel in FIG. 55 shows the Western analysis of *L. monocytogenes* p60 secretion using a polyclonal α -p60 antibody. The results demonstrate that equivalent amounts of Lm-secreted protein were loaded on the gel.

[0581] The results demonstrate that p60 can be used as a molecular chaperone to secrete heterologous proteins and facilitate presentation to the MHC class I pathway.

Example 30

Additional Examples of Antigen Expression and Secretion by Recombinant *Listeria monocytogenes*

[0582] A. Expression of the Intracellular Domain (ICD) of EphA2 from a Bicistronic Construct Using a Non-Listerial Signal Peptide

[0583] FIG. 56 shows the Western blot analysis of the expression and secretion of the intracellular domain (ICD) of EphA2 from bicistronic messages using a non-Listerial, non-secA1 signal sequence.

[0584] EphA2 is a protein comprised of an extracellular domain (ECD) and an intracellular domain (ICD). *Listeria* Δ actA Δ inlB were engineered to express a bicistronic mRNAs, where the bicistronic mRNAs encoded the extracellular domain and intracellular domain of EphA2 as discrete polypeptides. All of the sequences encoding the signal sequences used in the constructs (*B. subtilis* phoD signal peptide, *B. anthracis* Protective Antigen signal peptide, and *L. lactis* Usp45 signal peptide) were codon-optimized for expression in *L. monocytogenes*. The sequences encoding the ECD and ICD domains were also codon-optimized for expression in *L. monocytogenes*. The Listerial promoter hly from the LLO gene was used as the promoter in these constructs.

[0585] The expression cassettes encoding the bicistronic mRNA were integrated into the *Listeria* genome using the integration vector pPL2. Western blot analysis of various bacterial fractions using standard techniques was used to detect and measure the accumulated intracellular EphA2 domain. The results demonstrated that the intracellular domain of EphA2 was expressed and secreted from bicistronic constructs using non-Listerial signal peptides encoded by codon-optimized sequences.

[0586] The expression constructs comprised: (1) a codon-optimized sequence encoding the *L. lactis* Usp45 secretory sequence operably (functionally) linked with the coding sequence for the extracellular domain of EphA2 (first polypeptide) and a codon-optimized sequence encoding the *B. subtilis* phoD secretory signal operably linked with an intracellular domain of EphA2 (second polypeptide) (lane 1); and (2) a codon-optimized sequence encoding the *B. anthracis* Protective Antigen secretory sequence operably linked with the coding sequence for the extracellular domain of EphA2 (first polypeptide) and a codon-optimized sequence encoding the *B. subtilis* phoD secretory sequence operably linked with the coding sequence for the intracellular domain of EphA2 (second polypeptide) (lanes 2-3 (two different clones); see description of construction of this expression cassette in Example 28, above). Control studies (lane 4) with the attenuated parent *Listeria* Δ actA Δ inlB strain demonstrated a variable amount of detectable cross reactivity in some control blots. Lanes 1-3 show a slow migrating band and a fast moving band, where the fast moving band corresponds to the intracellular domain (ICD). Expressed intracellular domain of EphA2 from all of the constructs (lanes 1-3) was observed in all three bacterial fractions. Lane 4 (control) shows only the slow migrating

band. Because no antibody was available for the extracellular domain, expression/secretion of the extracellular domain was not assayed.

[0587] B. Plasmid Based Expression and Secretion of Murine Mesothelin as a Function of N-Terminal Fusion With Various Codon-Optimized Signal Peptides

[0588] FIG. 57 shows plasmid based expression and secretion of murine mesothelin expressed from a codon-optimized mesothelin coding sequence using various signal peptides, including non-Listerial signal sequences and non-secA1 signal sequences. Plasmid based expression and secretion of murine mesothelin is shown as a function of N-terminal fusion with various signal peptides encoded by codon-optimized sequences. In all cases, the sequences encoding the signal peptides of the mesothelin fusion, proteins were codon-optimized as well as the murine mesothelin coding sequence was codon-optimized for expression in *L. monocytogenes*. Expression and secretion of murine mesothelin from *L. monocytogenes* was measured, where the *Listeria* harbored a pAM401 plasmid, and where the plasmid encoded the mesothelin. Various plasmid-based constructs were tested, where the signal sequence was varied. Western blots were performed with proteins recovered from the various fractions of secreted proteins (A), the cell wall (B), and the cell lysate (C). For each fraction, lanes 1-2 show murine mesothelin expressed as a fusion with the *B. anthracis* Protective Antigen signal sequence, lanes 3-4 show murine mesothelin expressed as a fusion with the *Lactococcus lactis* Usp45 signal sequence, lanes 5-6 show murine mesothelin expressed as a fusion with the *B. subtilis* phoD signal sequence, lanes 7-8 show murine mesothelin expressed as a fusion with the p60 signal sequence, lanes 9-10 show murine mesothelin expressed as a fusion with the LLO signal sequence, and lane 11 shows protein expressed by the control host *Listeria* Δ actA Δ inlB. The results demonstrate that the highest expression and secretion was found where the signal sequence comprised *B. anthracis* Protective Antigen signal sequence (lanes 1-2) and *B. subtilis* phoD signal sequence (lanes 5-6).

[0589] C. *Listeria monocytogenes* Chromosomal-Based Expression and Secretion of Human Mesothelin

[0590] FIG. 58 shows the Western blot analysis of *Listeria monocytogenes* chromosomal-based expression and secretion of human mesothelin in various bacterial cell fractions (i.e., secreted protein, cell wall, and lysate). Expression and secretion of human mesothelin was tested when fused to a non-Listerial secA1 and non-secA1 signal peptides. The *Listeria* bacteria tested were all Δ actA Δ inlB *Listeria* and were as follows: *Listeria* Δ actA Δ inlB (control *Listeria* that was not engineered to express mesothelin) (Lane 1); *Listeria* encoding *B. anthracis* Protective Antigen signal sequence fused to Δ SS/ Δ GPI hMesothelin (Lanes 2-3); *Listeria* encoding *B. subtilis* phoD signal sequence fused to Δ SS/ Δ GPI hMesothelin (Lanes 4-5); *Listeria* encoding *B. anthracis* Protective Antigen signal sequence fused with full-length hMesothelin (Lanes 6-7); *Listeria* encoding *B. subtilis* phoD signal sequence fused to full-length hMesothelin (Lanes 8-9).

[0591] The sequences encoding the signal sequences fused to mesothelin in all of the above *Listeria* were codon-optimized for expression in *L. monocytogenes*. In addition, the mesothelin coding sequences (Δ SS/ Δ GPI and full-

length) were codon-optimized for expression in *L. monocytogenes* in each of the constructs. In each of the above *Listeria* expressing mesothelin, the mesothelin expression cassettes were inserted in the *Listeria* chromosome via integration with pPL2.

[0592] Highest expression occurred with the *B. subtilis* phoD secretory sequence where human mesothelin was engineered to delete its signal sequence and to delete a hydrophobic region (gpi region) (Lanes 4-5).

Example 31

Additional Examples of Immunogenicity and Anti-Tumor Efficacy of Recombinant *Listeria monocytogenes* Vaccines

[0593] The following examples disclose results of vaccination with the *Listeria* of the present invention, e.g., vaccine-dependent stimulation of cytokine expression, vaccine-dependent survival of an animal with tumors, vaccine-dependent reduction in tumor metastasis, and vaccine-dependent reduction in tumor volume.

[0594] A. Immunogenicity of *Listeria* Vaccine Comprising P-60-Model Antigen Chimera

[0595] FIG. 59A and B show delivery of a heterologous antigen to the MHC Class I pathway by *Listeria* expressing either a p60-antigen chimera or an LLO signal peptide-antigen fusion protein. The heterologous antigen used in this experiment was AH1-A5. Vaccination was with *Listeria* engineered to comprise a p60 protein chimera expression cassette encoding AH1-A5 (fused to the OVA SL8 peptide) inserted within the p60 polypeptide sequence including the N-terminal p60 signal peptide sequence ("p60-based construct"), or *Listeria* engineered to encode an LLO signal peptide linked to a nucleic acid encoding the same antigen, AH1-A5 embedded within OVA ("LLO-based construct"). Both of these constructs used the Listerial promoter hly. p60 is a Listerial peptidoglycan autolysin that is secreted by a secA2 pathway, while LLO is listeriolysin.

[0596] To generate the p60-based construct, the nucleic acid encoding p60 was engineered to contain a PstI cloning site, where the PstI cloning site represented a silent mutation, i.e., resulting in no change in the encoded amino acid sequence. The PstI site was located between the N-terminal signal sequence and the first of two LysM cell wall binding domains in the p60 sequence. A polynucleotide encoding a heterologous polypeptide comprising the AH1-A5 epitope (SPSYAYHQF (SEQ ID NO:73)) and SL8 epitope (SHNFEKL (SEQ ID NO:123)) was inserted in frame into the PstI cloning site. The coding sequences for these epitopes were separated by a unique XhoI site and codon-optimized for expression in *L. monocytogenes*. The insertion into the PstI site occurred at the equivalent of nucleotide base number 199 of p60. The first 1-70 amino acids of the p60 coding sequence were codon-optimized for expression in *L. monocytogenes*. Accordingly, the first 27 amino acids corresponding to the signal peptide were expressed from optimal codons for expression in *L. monocytogenes*. The antigen expression cassette further contained unique 5' and 3' KpnI and SacI sites, respectively for insertion into the MCS of the pPL2 plasmid, for site-specific integration adjacent to the trnA⁴ gene of the *L. monocytogenes* genome. The LLO-based construct comprised a sequence encoding an LLO

signal sequence operably linked to a nucleic acid encoding AH1-A5 within OVA (without use of any codon-optimization). Thus, in the present study, the signal peptide was either from *Listeria* LLO or from *Listeria* p60.

[0597] The constructs were placed into pPL2, a vector that mediates site-specific recombination with *Listeria* genome, and inserted into the *Listeria* genome.

[0598] FIG. 59A and B show the immune response to a vaccination (tail vein) of *Listeria* expressing the AH1-A5 antigen with p60 signal sequence/autolysin as a p60 chimera, and immune response to vaccination of *Listeria* expressing AH1-A5 antigen linked with the LLO signal sequence. In the x-axis of the figure, "Unstim" means that no peptide was added to the wells (i.e., the cells were unstimulated), while "AH1" means that the AH1 nonapeptide was added to the wells, and "AH1-A5" means that the AH1-A5 nonapeptide was added to the wells. All bacterial vaccines were engineered to contain an integrated nucleic acid encoding AH1-A5 (the bacterial vaccines did not encode AH1) (see, e.g., Slansky, et al. (2000) Immunity 13:529-538). Where the vaccination was done with the *Listeria* comprising the p60-based constructs, the strain is indicated on the x-axis of the figure as "p60." Where the vaccination was done with *Listeria* comprising the LLO-based constructs, the strain is indicated on the x-axis of the figure as "LLO."

[0599] The overall protocol for vaccination with *Listeria* expressing the P60-based construct was as follows: (1) Mice were vaccinated with *Listeria* (tail vein (i.v.)) containing an integrated nucleic acid, where the integrated nucleic acid encoded p60 containing a nucleic acid encoding AH1-A5 inserted at nucleotide 199 of p60. In other words, the nucleic acid encoding AH1-A5 antigen was in frame with and operably linked with p60 signal sequence and with p60 autolysin. The nucleic acid encoding AH1-A5 was codon optimized for expression in *L. monocytogenes*; (2) Seven days post infection, the spleens were removed; (3) Spleen cells were dissociated, placed in wells, and the spleen cells were incubated with either no added peptide (FIGS. 59A and 59B), with added AH1 (FIG. 59A), or with added AH1-A5 (FIG. 59B), as indicated on the x-axis; (4) After adding the peptide, cells were incubated for five hours, followed by assessment of the percent of IFN γ expressing CD8⁺ T cells by FACS analysis. An analogous protocol was used for vaccination with *Listeria* expressing the LLO-based construct.

[0600] The results demonstrate that the *Listeria* vaccines stimulated CD8⁺ T cell expression of IFN γ , where the added peptide was AH1 (FIG. 59A) or where the added peptide was AH1-A5 (FIG. 59B). Stimulation was somewhat higher where integrated AH1-A5 was operably linked with LLO signal sequence, and stimulation was somewhat lower when integrated AH1-A5 was operably linked with p60 signal sequence (FIGS. 59A and B).

[0601] FIGS. 60A and B show experiments conducted with the same two *Listeria* vaccines as described above, i.e., as shown in FIGS. 59A and B. FIG. 60A shows results where mice were vaccinated with the *Listeria* engineered to contain the p60-based construct ("p60") or with the *Listeria* engineered to contain the LLO-based construct ("LLO"). As indicated on the x-axis of FIG. 60A, the cell based assays were supplemented with no peptide (unstimulated; "unstim") or with LLO₉₁₋₉₉ peptide ("LLO91"; Badovinac

and Harty (2000) J. Immunol. 164:6444-6452). The results demonstrated a similar immune response (IFN γ expression) where the *Listeria* vaccine contained the p-60 based construct or the LLO-based construct. The stimulated immune response in FIG. 60A, as reflected in the results from the cell-based assay, is due to the *Listeria*'s endogenous expression of native LLO.

[0602] FIG. 60B shows results where mice were vaccinated with *Listeria* engineered to contain the p60-based construct, where the hly promoter and signal peptide sequences were operably linked with a nucleic acid encoding AH1-A5, or with *Listeria* engineered to contain the LLO-based construct, where the hly promoter and signal peptide were operably linked with a nucleic acid encoding AH1-A5. The added peptides were either no peptide (unstimulated; "unstim") or p60₂₁₇₋₂₂₅ ("p60-217"; Sijts, et al. (1997) J. Biol. Chem. 272:19261-19268), as indicated on the x-axis. The stimulated immune response in FIG. 60B, as reflected in the results from the cell based assay, is due to the *Listeria*'s expression of endogenous p60 for the LLO-based construct and the combination of endogenous p60 and the expressed p60 protein chimera sequence for the p60-based construct.

[0603] B. Therapeutic Efficacy of *Listeria* Expressing Human Mesothelin

[0604] The results depicted in FIG. 61 reveal that vaccination with *Listeria* expressing human mesothelin (huMesothelin) prolongs survival in tumor-bearing mice, where the tumor cells in the mice had been engineered to express human mesothelin. The tumor cells were CT26 cells expressing human mesothelin and the mice were Balb/c mice. (All CT26 tumor studies described herein involved Balb/c mice.) In one of the expression cassettes, a sequence encoding a non-*Listeria* signal sequence was operably linked in frame with a codon-optimized sequence encoding human mesothelin (deleted of its signal sequence and GPI anchor). The expression cassette encoding a signal peptide fused with human mesothelin (Δ GPI Δ SS) was administered to tumor-bearing mice in a *Listeria* vaccine in studies on the effect of the fusion protein on immune response to tumors. The expression cassette encoding the mesothelin fusion protein had been integrated into the *Listeria* chromosome. On Day 0, 2×10^5 CT26 cells expressing human mesothelin (CT26 huMeso+) were injected intravenously into the Balb/c mice. Vaccination of the mice was in the tail vein (i.v.). Inoculation with 1×10^7 colony forming units (CFU) *Listeria* (i.v.) occurred at day 3.

[0605] FIG. 61 shows the percent survival (shown on y-axis) of the mice to CT26 tumor expressing human mesothelin, where the vaccine comprises Hank's Balanced Salt Solution (HBSS) (a sham vaccine; "HBSS"); *Listeria* Δ actA Δ inlB expressing SF-AH1A5 from an integrated expression cassette (positive control vaccine; "SF-AH1A5"); or *Listeria* Δ actA Δ inlB comprising an expression cassette encoding *B. anthracis* Protective Antigen signal sequence (encoded by a non-codon optimized sequence) fused with huMesothelin (encoded by a codon-optimized sequence), where the huMesothelin had a deleted signal sequence and a deleted region encoding the hydrophobic gpi-anchoring peptide ("BaPA-huMeso Δ gpi Δ SS"). *Listeria* bearing the SF-AH1A5 construct and the BaPA-huMeso Δ gpi Δ SS construct contained these constructs as chromo-

somally integrated constructs. The nucleic acid molecule encoding SF-AH1A5 and the nucleic acid molecule encoding the BaPA-huMeso Δ gpi Δ SS construct had been integrated into the *Listeria* genome using pPL2. SF is shorthand for an eight amino acid peptide derived from ovalbumin, also known as SL8 (see, e.g., Shastri and Ganzalez (1993) J. Immunol. 150:2724-2736). The abbreviations "SF-AH1A5," "SF-AH1-A5," and "OVA/AH1-A5" refer to AH1-A5 connected to an ovalbumin scaffold. "SF AH1-A5" refers to the AH1-A5 (SPSYAYHQF (SEQ ID NO:73)) and the SF peptide fused to the N-terminus of amino acids 138 to 386 of GenBank Accession. No. P01012 (ovalbumin). The polynucleotides encoding "SF-AH1A5," in this example, comprised a codon-optimized nucleic acid encoding AH1-A5 and a non-codon optimized nucleic acid encoding the ovalbumin-derived sequence.

[0606] The results demonstrate that a single immunization with *Listeria* expressing huMesothelin prolongs survival of mice containing huMesothelin-expressing tumors. The survival percentage was highest with the chromosomally integrated *B. anthracis* Protective Antigen signal sequence fused with the Δ signal sequence/ Δ gpi huMesothelin (BaPA-huMeso Δ gpi Δ SS; closed squares). Survival was lowest where "vaccination" was with the control salt solution.

[0607] C. Reduction in Lung Tumor Nodule Level in Tumor-Bearing Mice Vaccinated With *Listeria* Expressing Human Mesothelin Due to Mesothelin-Specific Anti-Tumor Efficacy

[0608] The data in FIG. 62 demonstrate that the level of lung tumor nodules is reduced by vaccination with *Listeria* Δ actA Δ inlB expressing human mesothelin, where the tumor cells were engineered to express human mesothelin. The mouse strain was Balb/c and the lung tumor cells were CT26 cells harboring a vector expressing human mesothelin. On Day 0, 2×10^5 CT26 cells expressing human mesothelin were administered intravenously to the Balb/c mice. Sequences encoding various signal sequences were operably linked in frame with codon-optimized sequences encoding human mesothelin in expression cassettes. The expression cassettes encoding various signal peptides fused with human mesothelin were administered to the tumor-bearing mice via *Listeria* vaccines comprising the expression cassettes. On Day 3, 1×10^7 CFU/100 μ L of the *Listeria* vaccines were administered to the tumor-bearing mice intravenously. Negative control vaccinations were with HBSS or *Listeria* Δ actA Δ inlB. Positive control vaccinations were with *Listeria* expressing an OVA fusion protein comprising AH1A5 (in frame with the OVA sequence). (The OVA fusion protein comprising AH1A5 was encoded by a non-codon optimized expression cassette.) On Day 19, the mice were sacrificed, their lungs harvested, and the lung tumor nodules counted.

[0609] The *Listeria* vaccines reduced the number of metastases in the lungs. Control vaccines involving only HBSS or *Listeria* Δ actA Δ inlB resulted in a detected consistent 250 metastases per lung and an average of 135 metastasis per lung, respectively. *Listeria* bearing plasmid (pAM401) encoding LLO signal peptide fused to human mesothelin ("pAM-LLO-HuMeso") showed about 25 metastases per lung. The polynucleotide sequences of the pAM-LLO-HuMeso plasmid that encoded the LLO signal peptide and the human mesothelin sequence were codon-optimized for expression in *L. monocytogenes*. *Listeria*

bearing integrated sequences encoding *B. anthracis* Protective Antigen signal sequence (BaPA) fused with huMesothelin (Δ gpi/ Δ signal sequence) ("BaPA-HuMeso Δ gpi Δ ss") also showed on the average about 25 metastases per lung on average. The polynucleotide in BaPA-HuMeso Δ gpi Δ ss that encoded the *B. anthracis* Protective Antigen signal sequence was not codon-optimized, whereas the polynucleotide that encoded the human mesothelin sequence deleted of the mesothelin signal peptide and GPI anchor was codon-optimized for expression in *L. monocytogenes*.

[0610] FIG. 63 shows the results of a control study using mice comprising lung tumor nodules generated using CT26 parental target cells. Balb/c mice were used, but wt CT26 was instead injected (2×10^5 cells (i.v.) on Day 0). The study demonstrates that the anti-tumor efficacy of vaccination with the *Listeria* vaccine expressing mesothelin fusion proteins is mesothelin specific. Sequences encoding various signal sequences were operably linked in frame with codon-optimized sequences encoding human mesothelin in expression cassettes. (The constructs used in this experiment were identical to those used in the experiments above to generate the data shown in FIG. 62.) The expression cassettes encoding various signal peptides fused with human mesothelin were administered to the tumor-bearing mice via *Listeria* vaccines comprising the expression cassettes. Vaccination was in the tail vein (1×10^7 CFU/100 μ L i.v. on Day 3). In this particular study, the tumor cells did not express human mesothelin. Survival was determined. Where the data was available, the number of lung metastases was also measured. There were a total of five mice in each vaccination group. Negative control inoculation involved HBSS or *Listeria* Δ actA Δ inlB. Positive control inoculation involved *Listeria* expressing an OVA fusion comprising AH1A5 (not codon-optimized).

[0611] The results are shown in FIG. 63. Crosses indicate failure to survive and each vaccination group contained 5 mice. With the positive control inoculation, the mice survived, and the number of detected metastases in the lung was on the average about 25 per lung. As the tumor cells were not engineered to express human mesothelin, the mice inoculated with *Listeria* harboring a plasmid expressing LLO signal peptide fused with human mesothelin ("pAM-LLO-HuMeso") did not survive. Where mice were inoculated with *Listeria* bearing chromosomally integrated *B. anthracis* Protective Antigen secretory sequence (BaPA; encoded by a non-codon optimized nucleotide sequence) fused with with human mesothelin (Δ gpi/ Δ signal sequence) ("BaPA-HuMeso Δ gpi Δ ss"), some survived but others failed to survive.

[0612] D. Vaccination With *Listeria* Expressing Codon-Optimized Human Mesothelin Reduces Tumor Volume

[0613] FIG. 64 shows vaccination with *Listeria* (Δ actA Δ inlB) expressing human mesothelin from expression cassettes comprising codon-optimized mesothelin codon sequences reduces tumor volume.

[0614] Sequences encoding various signal sequences were operably linked in frame with codon-optimized sequences encoding human mesothelin in expression cassettes. The expression cassettes encoding various signal peptides fused with human mesothelin were administered to tumor-bearing mice via *Listeria* vaccines comprising the expression cassettes. The *Listeria* vaccines expressing human mesothelin that were used for vaccination of the tumor-bearing mice in

this study include the following: *Listeria* (Δ actA Δ inlB *L. monocytogenes*) bearing a pAM401 plasmid expressing and secreting LLO signal peptide (encoded by a sequence codon-optimized for expression in *L. monocytogenes*) fused with human mesothelin ("pAM opt.LLO-opt.huMeso"); *Listeria* bearing a pAM401 plasmid expressing *B. anthracis* Protective Antigen signal sequence (encoded by a non-codon optimized expression cassette) fused with huMesothelin ("pAM non-opt.BaPA-opt.huMeso"); and *Listeria* comprising an integrated expression cassette encoding *B. anthracis* Protective Antigen signal peptide (encoded by a non-codon optimized sequence) fused with huMesothelin, where the huMesothelin had a deleted signal sequence and a deleted region encoding the hydrophobic gpi-anchoring peptide ("Non-opt.BaPA-opt.huMeso delgpi-ss").

[0615] In the study, Balb/c mice were implanted subcutaneously with 2×10^5 cells of CT26 murine colon tumor cells engineered to expression human mesothelin (Day 0). Five mice were included in each vaccination group. On Day 3 following injection with the CT26 cells, the mice were vaccinated with non-*Listeria* control or 1×10^7 colony forming units (CFU) of the *Listeria* vaccine intravenously. Negative control inoculation involved HBSS. Positive control inoculation involved *Listeria* expressing SF-AH1A5 (codon optimized). (SF is an eight amino acid peptide derived from ovalbumin, also known as SL8 (see, e.g., Shastri and Ganzalez (1993) J. Immunol. 150:2724-2736).) At various time points, the mean tumor volume was determined.

[0616] The results of this study are shown in FIG. 64. The results demonstrated that vaccination with *Listeria* expressing human mesothelin fused to various signal peptides reduces tumor volume. Vaccination with *Listeria* expressing a *B. anthracis* Protective Antigen signal peptide fused with human mesothelin was protective (open circles with dotted line). Vaccination with *Listeria* expressing plasmid-encoded human mesothelin fused to LLO signal peptide was protective (open triangles). Vaccination with *Listeria* comprising a chromosomally integrated expression cassette encoding *B. anthracis* Protective Antigen (non-codon optimized nucleic acid) signal peptide fused with human mesothelin (Δ gpi/ Δ signal sequence) (open ovals with solid line) was also protective. Regarding the positive controls, *Listeria* expressing chromosomally integrated SF-AH1A5 (open squares) were also protective. The highest tumor volume, and earliest time of tumor growth onset, occurred in mice receiving the sham vaccine (HBSS).

[0617] E. Immunogenicity of a *Listeria* Vaccine Expressing Human Mesothelin Fused to a Non-*Listeria* Signal Sequence

[0618] FIG. 65 depicts the immunogenicity of a *Listeria* Δ actA Δ inlB-hMesothelin strain, where the *Listeria* contained a chromosomally integrated nucleic acid encoding hMesothelin fused to a *Bacillus anthracis* signal peptide (optimized Ba PA hMeso Δ GPI Δ SS). ELISPOT assays were used to assess immune response, where the assays were sensitive to expression of interferon-gamma.

[0619] The study comprised the following steps: (1) Mice (Balb/c mice or C57BL/6 mice) were vaccinated (i.v.) with the *Listeria* comprising an integrated expression cassette encoding *B. anthracis* Protective Antigen signal peptide (encoded by a non-codon optimized sequence) fused with huMesothelin (encoded by a codon-optimized sequences in

which the mesothelin signal sequence and hydrophobic gpi-anchoring sequences had been deleted); (2) After 7 days, the spleens were removed; (3) The cells removed from the spleens were dispersed in wells. Each well received about 200,000 spleen cells; (4) One of three kinds of medium were added to the wells, as indicated. Spleen cells from studies with Balb/c mice received medium only ("Unstimulated"), mesothelin peptide pool ("Meso pool"), or p60₂₁₇₋₂₂₅ ("p60₂₁₇"). Spleen cells from studies with C57BL/6 received medium only ("Unstimulated"), mesothelin peptide pool ("Meso pool"), or LLO₂₉₆₋₃₀₄ ("LLO₂₉₆₋₃₀₄"); (5) ELISPOT assays were performed to determine number of immune cells responding to the added peptide(s). The mesothelin peptide pool comprised 153 different peptides, where these peptides spanned the entire sequence of hMesothelin, where each peptide was 15 amino acids long, overlapping the adjacent peptides by 11 amino acids.

[0620] The results of the ELISPOT assays are shown in FIG. 65. The results indicated that the *Listeria* vaccine

expressing human mesothelin fused to *B. anthracis* signal peptide was capable of inducing an immune response to mesothelin in Balb/c mice. A higher IFN-gamma response to *Listeria*-expressed hMesothelin was observed with the Balb/c mouse immune system than with the C57BL/6 immune system. ELISPOT signal to p60 or LLO was in response to the *Listeria*'s naturally occurring p60 and LLO proteins.

[0621] All publications, patents, patent applications, internet sites, and accession numbers/database sequences (including both polynucleotide and polypeptide sequences) cited herein are hereby incorporated by reference herein in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, internet site, or accession number/database sequence were specifically and individually indicated to be so incorporated by reference.

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 65            70            75            80
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 770 775 780
 Gly Met Lys Tyr Leu Ala Asn Met Asn Tyr Val His Arg Asp Leu Ala
 785 790 795 800
 Ala Arg Asn Ile Leu Val Asn Ser Asn Leu Val Cys Lys Val Ser Asp
 805 810 815
 Phe Gly Leu Ser Arg Val Leu Glu Asp Asp Pro Glu Ala Thr Tyr Thr
 820 825 830
 Thr Ser Gly Lys Ile Pro Ile Arg Trp Thr Ala Pro Glu Ala Ile
 835 840 845
 Ser Tyr Arg Lys Phe Thr Ser Ala Ser Asp Val Trp Ser Phe Gly Ile
 850 855 860
 Val Met Trp Glu Val Met Thr Tyr Gly Glu Arg Pro Tyr Trp Glu Leu
 865 870 875 880
 Ser Asn His Glu Val Met Lys Ala Ile Asn Asp Gly Phe Arg Leu Pro
 885 890 895
 Thr Pro Met Asp Cys Pro Ser Ala Ile Tyr Gln Leu Met Met Gln Cys
 900 905 910
 Trp Gln Gln Glu Arg Ala Arg Arg Pro Lys Phe Ala Asp Ile Val Ser
 915 920 925
 Ile Leu Asp Lys Leu Ile Arg Ala Pro Asp Ser Leu Lys Thr Leu Ala
 930 935 940
 Asp Phe Asp Pro Arg Val Ser Ile Arg Leu Pro Ser Thr Ser Gly Ser
 945 950 955 960
 Glu Gly Val Pro Phe Arg Thr Val Ser Glu Trp Leu Glu Ser Ile Lys
 965 970 975
 Met Gln Gln Tyr Thr Glu His Phe Met Ala Ala Gly Tyr Thr Ala Ile
 980 985 990
 Glu Lys Val Val Gln Met Thr Asn Asp Asp Ile Lys Arg Ile Gly Val
 995 1000 1005
 Arg Leu Pro Gly His Gln Lys Arg Ile Ala Tyr Ser Leu Leu Gly Leu
 1010 1015 1020
 Lys Asp Gln Val Asn Thr Val Gly Ile Pro Ile
 1025 1030 1035

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<210> SEQ ID NO 5
 <211> LENGTH: 1506
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

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cagggaagg aagtggtact gctggacttt gctgcagctg gaggggagct cggctggctc   60
acacacccgt atggcaaagg gtgggacctg atgcagaaca tcatgaatga catgccgatc   120
tacatgtact ccgtgtgcaa cgtgatgtct ggcgaccagg acaactggct ccgcaccaac   180
tgggtgtacc gaggaagagg tgagcgtatc ttcattgagc tcaagtttac tgtacgtgac   240
tgcaacagct tccctggagg cgccagctcc tgcaaggaga ctttcaacct ctactatgcc   300
gagtcggacc tggactacgg caccaacttc cagaagcgcc tgttcaccaa gattgacacc   360
attgcgcccg atgagatcac cgtcagcagc gacttcgagg cagccaccgt gaagctgaac   420
gtggaggagc gctccgtggg gccgctcacc cgcaaaggct tctacctggc cttccaggat   480
atcggtgccct gtgtggcgct gctctccgct cgtgtctact acaagaagtg ccccagagctg   540
ctgcaggggc tggcccaact ccctgagacc atcgccggct ctgatgcacc ttccctggcc   600
actgtggccg gcacctgtgt ggaccatgcc gtggtgccac cgggggggtga agagccccgt   660
atgcactgtg cagtggatgg cgagtggctg gtgcccattg ggcagtgcct gtgccaggca   720
ggctacgaga aggtggagga tgccctgccag gcctgctcgc ctggattttt taagtttgag   780
gcactctgaga gcccctgctt ggagtgcctt gagcacacgc tgccatcccc tgagggtgcc   840
acctcctgag agtgtgagga aggtctcttc cgggcacctc aggaaccagc gtcgatgcct   900
tgcacacgac cccctccgcg cccacactac ctcacagccg tgggcatggg tgccaaggty   960
gagctgcgct ggacgcccc tcaggacagc gggggccgag aggaacattgt ctacagcgctc  1020
acctgcgaac agtgcctggc cgagtctggg gaatgcgggc cgtgtgaggc cagtgtgcgc  1080
tactcggagc ctctcagcgg actgacccgc accagtgtga cagtgagcga cctggagccc  1140
cacatgaact acaccttcac cgtggaggcc cgcaatggcg tctcaggcct ggtaaccagc  1200
cgcagcttcc gtactgccag tgctcagcatc aaccagacag agccccccaa ggtgaggctg  1260
gagggccgca gcaccacctc gcttagcgct tcctggagca tccccccgcc gcagcagagc  1320
cgagtgtgga agtacgaggt cacttacccg aagaaggag actccaacag ctacaatgtg  1380
cgccgcaccg agggttttct cgtgacctg gacgacctgg cccagacac cacctacctg  1440
gtccaggtgc aggcactgac gcaggagggc cagggggccg gcagcagggt gcacgaattc  1500
cagacg                                           1506

```

<210> SEQ ID NO 6
 <211> LENGTH: 1506
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 6

```

caaggtaaag aagttgtttt attagatttt gcagcagcag gtggtgaatt aggttggtta   60
acacatccat atggtaaagg ttgggattta atgcaaaata ttatgaatga tatgccaatt   120
tatatgtata gtgtttgtaa tgttatgagt ggtgatcaag ataattggtt acgtacaaat   180

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tgggtttatc	gtggtgaagc	agaacgtatt	tttattgaat	taaaatttac	agttcgtgat	240
tgtaatagtt	ttccaggtgg	tgcaagtagt	tgtaagaaa	catttaattt	atattatgca	300
gaaagtgatt	tagattatgg	tacaattttt	caaaaacggt	tatttacaaa	aattgataca	360
attgcaccag	atgaaattac	agttagtagt	gattttgaag	cacgtcatgt	taaattaaat	420
gttgaagaac	gtagtgttgg	tccattaaca	cgtaaagggt	tttatttagc	atttcaagat	480
attggtgcat	gtgttgcat	attaagtgtt	cgtgtttatt	ataaaaaatg	tccagaatta	540
ttacaagggt	tagcacattt	tccagaaaca	attgcaggta	gtgatgcacc	aagtttagca	600
acagttgcag	gtacatgtgt	tgatcatgca	gttggtccac	cagggtgtga	agaaccacgt	660
atgcattgtg	cagttgatgg	tgaatgggta	gttccaattg	gtcaatgttt	atgtcaagca	720
ggttatgaaa	aagttgaaga	tgcatgtcaa	gcattgtatc	caggtttttt	ttaatttgaa	780
gcaagtgaag	gtccatgttt	agaatgtcca	gaacatacat	taccaagtcc	agaaggtgca	840
acaagttgtg	aattggaaga	aggttttttt	cgtgcaccac	aagatccagc	aagtatgcca	900
tgtaacgctc	caccaagtgc	accacattat	ttaacagcag	ttggtatggg	tgcaaaagtt	960
gaattacggt	ggacaccacc	acaagatagt	ggtgggtcgt	aagatattgt	ttatagtgtt	1020
acatgtgaac	aattgtggcc	agaaagtggg	gaatgtgggc	catgtgaagc	aagtgttcgt	1080
tatatgaac	caccacatgg	tttaacacgt	acaagtgtta	cagttagtga	tttagaacca	1140
catatgaatt	atacatttac	agttgaagca	cgtaattggt	ttagtggttt	agttacaagt	1200
cgtagttttc	gtacagcaag	tgtttagtatt	aatcaaacag	aaccacacaa	agttcgttta	1260
gaaggtcgtg	gtacaacaag	tttaagtgtt	agttggagta	ttccaccacc	acaacaaagt	1320
cgtgttttga	aattgaaggt	tacatatcgt	aaaaaagggt	atagtaatag	ttataatgtt	1380
cgtcgtacag	aaggttttag	tgttacatta	gatgatttag	caccagatac	aacatattta	1440
gttcaagttc	aagcattaac	acaagaagggt	caaggtgcag	gtagtcgtgt	tcatgaattt	1500
caaacaca						1506

<210> SEQ ID NO 7

<211> LENGTH: 502

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

Gln	Gly	Lys	Glu	Val	Val	Leu	Leu	Asp	Phe	Ala	Ala	Ala	Gly	Gly	Glu
1				5					10					15	
Leu	Gly	Trp	Leu	Thr	His	Pro	Tyr	Gly	Lys	Gly	Trp	Asp	Leu	Met	Gln
			20					25					30		
Asn	Ile	Met	Asn	Asp	Met	Pro	Ile	Tyr	Met	Tyr	Ser	Val	Cys	Asn	Val
		35					40					45			
Met	Ser	Gly	Asp	Gln	Asp	Asn	Trp	Leu	Arg	Thr	Asn	Trp	Val	Tyr	Arg
	50				55					60					
Gly	Glu	Ala	Glu	Arg	Ile	Phe	Ile	Glu	Leu	Lys	Phe	Thr	Val	Arg	Asp
65			70					75					80		
Cys	Asn	Ser	Phe	Pro	Gly	Gly	Ala	Ser	Ser	Cys	Lys	Glu	Thr	Phe	Asn
			85					90					95		
Leu	Tyr	Tyr	Ala	Glu	Ser	Asp	Leu	Asp	Tyr	Gly	Thr	Asn	Phe	Gln	Lys
			100				105					110			
Arg	Leu	Phe	Thr	Lys	Ile	Asp	Thr	Ile	Ala	Pro	Asp	Glu	Ile	Thr	Val

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115	120	125
Ser Ser Asp Phe Glu Ala Arg His Val Lys Leu Asn Val Glu Glu Arg 130 135 140		
Ser Val Gly Pro Leu Thr Arg Lys Gly Phe Tyr Leu Ala Phe Gln Asp 145 150 155 160		
Ile Gly Ala Cys Val Ala Leu Leu Ser Val Arg Val Tyr Tyr Lys Lys 165 170 175		
Cys Pro Glu Leu Leu Gln Gly Leu Ala His Phe Pro Glu Thr Ile Ala 180 185 190		
Gly Ser Asp Ala Pro Ser Leu Ala Thr Val Ala Gly Thr Cys Val Asp 195 200 205		
His Ala Val Val Pro Pro Gly Gly Glu Glu Pro Arg Met His Cys Ala 210 215 220		
Val Asp Gly Glu Trp Leu Val Pro Ile Gly Gln Cys Leu Cys Gln Ala 225 230 235 240		
Gly Tyr Glu Lys Val Glu Asp Ala Cys Gln Ala Cys Ser Pro Gly Phe 245 250 255		
Phe Lys Phe Glu Ala Ser Glu Ser Pro Cys Leu Glu Cys Pro Glu His 260 265 270		
Thr Leu Pro Ser Pro Glu Gly Ala Thr Ser Cys Glu Cys Glu Glu Gly 275 280 285		
Phe Phe Arg Ala Pro Gln Asp Pro Ala Ser Met Pro Cys Thr Arg Pro 290 295 300		
Pro Ser Ala Pro His Tyr Leu Thr Ala Val Gly Met Gly Ala Lys Val 305 310 315 320		
Glu Leu Arg Trp Thr Pro Pro Gln Asp Ser Gly Gly Arg Glu Asp Ile 325 330 335		
Val Tyr Ser Val Thr Cys Glu Gln Cys Trp Pro Glu Ser Gly Glu Cys 340 345 350		
Gly Pro Cys Glu Ala Ser Val Arg Tyr Ser Glu Pro Pro His Gly Leu 355 360 365		
Thr Arg Thr Ser Val Thr Val Ser Asp Leu Glu Pro His Met Asn Tyr 370 375 380		
Thr Phe Thr Val Glu Ala Arg Asn Gly Val Ser Gly Leu Val Thr Ser 385 390 395 400		
Arg Ser Phe Arg Thr Ala Ser Val Ser Ile Asn Gln Thr Glu Pro Pro 405 410 415		
Lys Val Arg Leu Glu Gly Arg Ser Thr Thr Ser Leu Ser Val Ser Trp 420 425 430		
Ser Ile Pro Pro Pro Gln Gln Ser Arg Val Trp Lys Tyr Glu Val Thr 435 440 445		
Tyr Arg Lys Lys Gly Asp Ser Asn Ser Tyr Asn Val Arg Arg Thr Glu 450 455 460		
Gly Phe Ser Val Thr Leu Asp Asp Leu Ala Pro Asp Thr Thr Tyr Leu 465 470 475 480		
Val Gln Val Gln Ala Leu Thr Gln Glu Gly Gln Gly Ala Gly Ser Arg 485 490 495		
Val His Glu Phe Gln Thr 500		

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<211> LENGTH: 1689
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Fusion protein coding sequence

<400> SEQUENCE: 8
atgaaaaaaa taatgctagt ttttattaca cttatattag ttagtctacc aattgcgcaa    60
caaaactgaag caaaggatgc atctgcattc aataaagaaa attcaatttc atccatggca    120
ccaccagcat ctccgcctgc aagtcctaag acgccaatcg aaaagaaaca cgcggatctc    180
gagcagggca aggaagtggg actgctggac tttgctgcag ctggagggga gctcggctgg    240
ctcacacacc cgtatggcaa aggggtgggac ctgatgcaga acatcatgaa tgacatgccg    300
atctacatgt actccgtgtg caacgtgatg tctggcgacc aggacaactg gctccgcacc    360
aactgggtgt accgaggaga ggctgagcgt atcttcattg agctcaagtt tactgtacgt    420
gactgcaaca gcttccttgg tggcgccagc tcctgcaagg agactttcaa cctctactat    480
gccgagtcgg acctggacta cggcaccaac ttccagaagc gcctgttcac caagattgac    540
accattgcgc ccgatgagat caccgtcagc agcgacttcg aggcacgcc a cgtgaagctg    600
aacgtggagg agcgctccgt gggggcgcgc acccgcaaag gcttctacct ggcttccag    660
gatatcggtg cctgtgtggc gctgctctcc gtccgtgtct actacaagaa gtgccccgag    720
ctgctgcagg gcctggccca ctccctgag accatcgccg gctctgatgc accttccctg    780
gccactgtgg ccggcacctg tgtggaccat gccgtgtgtc caccgggggg tgaagagccc    840
cgtatgcact gtgcagtggg tggcgagtgg ctggtgcccc ttgggcagtg cctgtgccag    900
gcaggctacg agaagggtga gggatgcctg caggcctgct cgcctggatt ttttaagttt    960
gaggcatctg agagcccctg cttggagtgc cctgagcaca cgctgccatc ccctgagggg    1020
gccacctcct gcgagtgtga ggaaggcttc ttccgggcac ctccaggacc agcgtcgatg    1080
ccttgcaacg gacccccctc cgccccacac tacctcacag ccgtgggcat gggtgccaag    1140
gtggagctgc gctggagccc ccctcaggac agcggggggc gcgaggacat tgtctacagc    1200
gtcaactgcg aacagtgtg gcccgagtct ggggaatgcg gcccggtgtg ggccagtgtg    1260
cgctactcgg agcctcctca cggactgacc cgcaccagtg tgacagtgag cgacctggag    1320
ccccacatga actacacctt caccgtggag gcccgcaatg gcgtctcagg cctggtaacc    1380
agccgcagct tccgtactgc cagtgtcagc atcaaccaga cagagccccc caaggtgagg    1440
ctggaggggc gcagcaccac ctcgcttagc gtctcctgga gcaccccccc gccgcagcag    1500
agccgagtgt ggaagtacga ggtaacttac cgcaagaagg gagactccaa cagctacaat    1560
gtgcgccgca ccgagggttt ctccgtgacc ctggacgacc tggccccaga caccacctac    1620
ctggtccagg tgcaggcact gacgcaggag ggccaggggg ccggcagcag ggtgcacgaa    1680
ttccagacg                                     1689

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<210> SEQ ID NO 9
<211> LENGTH: 563
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 9

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-continued

Met	Lys	Lys	Ile	Met	Leu	Val	Phe	Ile	Thr	Leu	Ile	Leu	Val	Ser	Leu
1				5					10					15	
Pro	Ile	Ala	Gln	Gln	Thr	Glu	Ala	Lys	Asp	Ala	Ser	Ala	Phe	Asn	Lys
			20					25					30		
Glu	Asn	Ser	Ile	Ser	Ser	Met	Ala	Pro	Pro	Ala	Ser	Pro	Pro	Ala	Ser
	35						40					45			
Pro	Lys	Thr	Pro	Ile	Glu	Lys	Lys	His	Ala	Asp	Leu	Glu	Gln	Gly	Lys
	50					55					60				
Glu	Val	Val	Leu	Leu	Asp	Phe	Ala	Ala	Ala	Gly	Gly	Glu	Leu	Gly	Trp
65					70					75					80
Leu	Thr	His	Pro	Tyr	Gly	Lys	Gly	Trp	Asp	Leu	Met	Gln	Asn	Ile	Met
			85						90					95	
Asn	Asp	Met	Pro	Ile	Tyr	Met	Tyr	Ser	Val	Cys	Asn	Val	Met	Ser	Gly
			100					105					110		
Asp	Gln	Asp	Asn	Trp	Leu	Arg	Thr	Asn	Trp	Val	Tyr	Arg	Gly	Glu	Ala
	115						120					125			
Glu	Arg	Ile	Phe	Ile	Glu	Leu	Lys	Phe	Thr	Val	Arg	Asp	Cys	Asn	Ser
	130					135					140				
Phe	Pro	Gly	Gly	Ala	Ser	Ser	Cys	Lys	Glu	Thr	Phe	Asn	Leu	Tyr	Tyr
145					150					155					160
Ala	Glu	Ser	Asp	Leu	Asp	Tyr	Gly	Thr	Asn	Phe	Gln	Lys	Arg	Leu	Phe
			165					170						175	
Thr	Lys	Ile	Asp	Thr	Ile	Ala	Pro	Asp	Glu	Ile	Thr	Val	Ser	Ser	Asp
			180					185					190		
Phe	Glu	Ala	Arg	His	Val	Lys	Leu	Asn	Val	Glu	Glu	Arg	Ser	Val	Gly
	195					200						205			
Pro	Leu	Thr	Arg	Lys	Gly	Phe	Tyr	Leu	Ala	Phe	Gln	Asp	Ile	Gly	Ala
	210					215						220			
Cys	Val	Ala	Leu	Leu	Ser	Val	Arg	Val	Tyr	Tyr	Lys	Lys	Cys	Pro	Glu
225					230					235					240
Leu	Leu	Gln	Gly	Leu	Ala	His	Phe	Pro	Glu	Thr	Ile	Ala	Gly	Ser	Asp
			245						250					255	
Ala	Pro	Ser	Leu	Ala	Thr	Val	Ala	Gly	Thr	Cys	Val	Asp	His	Ala	Val
			260					265					270		
Val	Pro	Pro	Gly	Gly	Glu	Glu	Pro	Arg	Met	His	Cys	Ala	Val	Asp	Gly
		275					280					285			
Glu	Trp	Leu	Val	Pro	Ile	Gly	Gln	Cys	Leu	Cys	Gln	Ala	Gly	Tyr	Glu
	290					295						300			
Lys	Val	Glu	Asp	Ala	Cys	Gln	Ala	Cys	Ser	Pro	Gly	Phe	Phe	Lys	Phe
305					310					315					320
Glu	Ala	Ser	Glu	Ser	Pro	Cys	Leu	Glu	Cys	Pro	Glu	His	Thr	Leu	Pro
			325						330					335	
Ser	Pro	Glu	Gly	Ala	Thr	Ser	Cys	Glu	Cys	Glu	Glu	Gly	Phe	Phe	Arg
			340					345					350		
Ala	Pro	Gln	Asp	Pro	Ala	Ser	Met	Pro	Cys	Thr	Arg	Pro	Pro	Ser	Ala
		355					360						365		
Pro	His	Tyr	Leu	Thr	Ala	Val	Gly	Met	Gly	Ala	Lys	Val	Glu	Leu	Arg
	370					375						380			
Trp	Thr	Pro	Pro	Gln	Asp	Ser	Gly	Gly	Arg	Glu	Asp	Ile	Val	Tyr	Ser
385					390					395					400
Val	Thr	Cys	Glu	Gln	Cys	Trp	Pro	Glu	Ser	Gly	Glu	Cys	Gly	Pro	Cys

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405	410	415
Glu Ala Ser Val Arg Tyr Ser Glu Pro Pro His Gly Leu Thr Arg Thr		
420	425	430
Ser Val Thr Val Ser Asp Leu Glu Pro His Met Asn Tyr Thr Phe Thr		
435	440	445
Val Glu Ala Arg Asn Gly Val Ser Gly Leu Val Thr Ser Arg Ser Phe		
450	455	460
Arg Thr Ala Ser Val Ser Ile Asn Gln Thr Glu Pro Pro Lys Val Arg		
465	470	475
Leu Glu Gly Arg Ser Thr Thr Ser Leu Ser Val Ser Trp Ser Ile Pro		
485	490	495
Pro Pro Gln Gln Ser Arg Val Trp Lys Tyr Glu Val Thr Tyr Arg Lys		
500	505	510
Lys Gly Asp Ser Asn Ser Tyr Asn Val Arg Arg Thr Glu Gly Phe Ser		
515	520	525
Val Thr Leu Asp Asp Leu Ala Pro Asp Thr Thr Tyr Leu Val Gln Val		
530	535	540
Gln Ala Leu Thr Gln Glu Gly Gln Gly Ala Gly Ser Arg Val His Glu		
545	550	555
		560
Phe Gln Thr		

<210> SEQ ID NO 10
 <211> LENGTH: 1989
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette, encodes fusion protein

<400> SEQUENCE: 10

```

ggtacctcct ttgattagta ttttctatc ttaaagttac ttttatgtgg aggcattaac      60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata      120
atattgcgtt tcattcttag aagcgaattt cgccaatatt ataattatca aaagagaggg      180
gtggcaaacg gtatttggca ttattagggt aaaaaatgta gaaggagagt gaaacccatg      240
aaaaaaataa tgctagtgtt tattacactt atattagtta gtctaccaat tgcgcaacaa      300
actgaagcaa aggatgcata tgcattcaat aaagaaaatt caatttcata catggcacca      360
ccagcatctc cgcttgcaag tcctaagacg ccaatcgaaa agaaacacgc ggatggatcc      420
gattataaag atgatgatga taaacaaggt aaagaagttg ttttattaga tttgcagca      480
gcaggtgggt aattagggtg gttaacacat ccatatggta aaggttggga tttaatgcaa      540
aatattatga atgatatgcc aatttatatg tatagtgttt gtaatgttat gagtgggtgat      600
caagataatt ggttacgtac aaattggggt tatcgtgggt aagcagaacg tatttttatt      660
gaattaaaat ttacagttcg tgattgtaat agttttccag gtggtgcaag tagttgtaaa      720
gaaacattta atttatatta tgcagaaagt gatttagatt atggtacaaa ttttcaaaaa      780
cgtttattta caaaaattga tacaattgca ccagatgaaa ttacagttag tagtgatttt      840
gaagcacgtc atgttaaatt aaatgttgaa gaacgtagtg ttggtccatt aacacgtaaa      900
ggtttttatt tagcatttca agatatttgt gcattgtgtg cattattaag tgttcgtgtt      960
tattataaaa aatgtccaga attattacaa ggtttagcac attttccaga aacaattgca     1020
ggtagtgatg caccaagttt agcaacagtt gcaggatcat gtgttgatca tgcagttgtt     1080
  
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ccaccagggtg gtgaagaacc acgtatgcat tgtgcagttg atggtgaatg gttagttcca 1140
attggtcaat gtttatgtca agcagggttat gaaaaagttg aagatgcatg tcaagcatgt 1200
agtccagggtt tttttaaatt tgaagcaagt gaaagtccat gtttagaatg tccagaacat 1260
acattaccaa gtccagaagg tgcaacaagt tgtgaatgtg aagaaggttt ttttcgtgca 1320
ccacaagatc cagcaagtat gccatgtaca cgtccaccaa gtgcaccaca ttatttaaca 1380
gcagttggta tgggtgcaaa agttgaatta cgttggtcac caccacaaga tagtgggtgt 1440
cgtgaagata ttgtttatag tgttacatgt gaacaatgtt ggccagaaag tggagaatgt 1500
ggtccatgtg aagcaagtgt tcgttatagt gaaccaccac atgggttaac acgtacaagt 1560
gttacagtta gtgatttaga accacatatg aattatacat ttacagtga agcacgtaat 1620
ggtgttagtg gtttagttac aagtcgtagt tttcgtacag caagtgttag tattaatcaa 1680
acagaaccac caaaagtctg tttagaaggt cgtagtacaa caagtttaag tgtagttgg 1740
agtattccac caccacaaca aagtcgtgtt tggaaatatg aagttacata tcgtaaaaaa 1800
ggtgatagta atagttataa tgttcgtcgt acagaagggt ttagtggtac attagatgat 1860
ttagcaccag atacaacata ttagttcaa gttcaagcat taacacaaga aggtcaagggt 1920
gcaggtagtc gtgttcatga atttcaaaac gaacaaaaat taattagtga agaagattta 1980
tgagagctc 1989

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<210> SEQ ID NO 11

<211> LENGTH: 581

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 11

```

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu
 1             5             10            15
Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys
          20             25            30
Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser
          35             40            45
Pro Lys Thr Pro Ile Glu Lys Lys His Ala Asp Gly Ser Asp Tyr Lys
          50             55            60
Asp Asp Asp Asp Lys Gln Gly Lys Glu Val Val Leu Leu Asp Phe Ala
          65             70            75            80
Ala Ala Gly Gly Glu Leu Gly Trp Leu Thr His Pro Tyr Gly Lys Gly
          85             90            95
Trp Asp Leu Met Gln Asn Ile Met Asn Asp Met Pro Ile Tyr Met Tyr
          100            105           110
Ser Val Cys Asn Val Met Ser Gly Asp Gln Asp Asn Trp Leu Arg Thr
          115            120           125
Asn Trp Val Tyr Arg Gly Glu Ala Glu Arg Ile Phe Ile Glu Leu Lys
          130            135           140
Phe Thr Val Arg Asp Cys Asn Ser Phe Pro Gly Gly Ala Ser Ser Cys
          145            150           155           160
Lys Glu Thr Phe Asn Leu Tyr Tyr Ala Glu Ser Asp Leu Asp Tyr Gly
          165            170           175

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Thr	Asn	Phe	Gln	Lys	Arg	Leu	Phe	Thr	Lys	Ile	Asp	Thr	Ile	Ala	Pro
			180												
Asp	Glu	Ile	Thr	Val	Ser	Ser	Asp	Phe	Glu	Ala	Arg	His	Val	Lys	Leu
			195												
Asn	Val	Glu	Glu	Arg	Ser	Val	Gly	Pro	Leu	Thr	Arg	Lys	Gly	Phe	Tyr
			210												
Leu	Ala	Phe	Gln	Asp	Ile	Gly	Ala	Cys	Val	Ala	Leu	Leu	Ser	Val	Arg
			225	230			235			240					
Val	Tyr	Tyr	Lys	Lys	Cys	Pro	Glu	Leu	Leu	Gln	Gly	Leu	Ala	His	Phe
			245	250			255								
Pro	Glu	Thr	Ile	Ala	Gly	Ser	Asp	Ala	Pro	Ser	Leu	Ala	Thr	Val	Ala
			260	265			270								
Gly	Thr	Cys	Val	Asp	His	Ala	Val	Val	Pro	Pro	Gly	Gly	Glu	Glu	Pro
			275	280			285								
Arg	Met	His	Cys	Ala	Val	Asp	Gly	Glu	Trp	Leu	Val	Pro	Ile	Gly	Gln
			290	295			300								
Cys	Leu	Cys	Gln	Ala	Gly	Tyr	Glu	Lys	Val	Glu	Asp	Ala	Cys	Gln	Ala
			305	310			315			320					
Cys	Ser	Pro	Gly	Phe	Phe	Lys	Phe	Glu	Ala	Ser	Glu	Ser	Pro	Cys	Leu
			325	330			335								
Glu	Cys	Pro	Glu	His	Thr	Leu	Pro	Ser	Pro	Glu	Gly	Ala	Thr	Ser	Cys
			340	345			350								
Glu	Cys	Glu	Glu	Gly	Phe	Phe	Arg	Ala	Pro	Gln	Asp	Pro	Ala	Ser	Met
			355	360			365			370					
Pro	Cys	Thr	Arg	Pro	Pro	Ser	Ala	Pro	His	Tyr	Leu	Thr	Ala	Val	Gly
			370	375			380			385					
Met	Gly	Ala	Lys	Val	Glu	Leu	Arg	Trp	Thr	Pro	Pro	Gln	Asp	Ser	Gly
			385	390			395			400					
Gly	Arg	Glu	Asp	Ile	Val	Tyr	Ser	Val	Thr	Cys	Glu	Gln	Cys	Trp	Pro
			405	410			415			420					
Glu	Ser	Gly	Glu	Cys	Gly	Pro	Cys	Glu	Ala	Ser	Val	Arg	Tyr	Ser	Glu
			420	425			430			435					
Pro	Pro	His	Gly	Leu	Thr	Arg	Thr	Ser	Val	Thr	Val	Ser	Asp	Leu	Glu
			435	440			445			450					
Pro	His	Met	Asn	Tyr	Thr	Phe	Thr	Val	Glu	Ala	Arg	Asn	Gly	Val	Ser
			450	455			460			465					
Gly	Leu	Val	Thr	Ser	Arg	Ser	Phe	Arg	Thr	Ala	Ser	Val	Ser	Ile	Asn
			465	470			475			480					
Gln	Thr	Glu	Pro	Pro	Lys	Val	Arg	Leu	Glu	Gly	Arg	Ser	Thr	Thr	Ser
			485	490			495			500					
Leu	Ser	Val	Ser	Trp	Ser	Ile	Pro	Pro	Pro	Gln	Gln	Ser	Arg	Val	Trp
			500	505			510			515					
Lys	Tyr	Glu	Val	Thr	Tyr	Arg	Lys	Lys	Gly	Asp	Ser	Asn	Ser	Tyr	Asn
			515	520			525			530					
Val	Arg	Arg	Thr	Glu	Gly	Phe	Ser	Val	Thr	Leu	Asp	Asp	Leu	Ala	Pro
			530	535			540			545					
Asp	Thr	Thr	Tyr	Leu	Val	Gln	Val	Gln	Ala	Leu	Thr	Gln	Glu	Gly	Gln
			545	550			555			560					
Gly	Ala	Gly	Ser	Arg	Val	His	Glu	Phe	Gln	Thr	Glu	Gln	Lys	Leu	Ile
			565	570			575			580					
Ser	Glu	Glu	Asp	Leu											

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580

<210> SEQ ID NO 12
<211> LENGTH: 1989
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette, encodes fusion protein

<400> SEQUENCE: 12

ggtagctcct ttgattagta tttcctatc ttaaagttac ttttatgtgg aggcattaac	60
atgtgttaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata	120
atattgcgtt tcattcttag aagcgaattt cgccaatatt ataattatca aaagagaggg	180
gtggcaaacg gtatttggca ttattagggt aaaaaatgta gaaggagagt gaaacccatg	240
aaaaaaaaa ttgttagttt tattacatta attttagtta gtttaccat tgcacaacaa	300
acagaagcaa aagatgcaag tgcatttaaa aaagaaaata gtattagtag tatggcacca	360
ccagcaagtc caccagcaag tccaaaaaca ccaattgaaa aaaaacatgc agatggatcc	420
gattataaag atgatgatga taaacaaggt aaagaagttg ttttattaga ttttgagca	480
gcaggtggtg aattagggtg gttaacacat ccattatgga aaggttggga tttaatgcaa	540
aattattatga atgatatgcc aatttatatg tatagtgttt gtaagtgtat gagggtgat	600
caagataatt ggttacgtac aaattgggtt tatcgtggtg aagcagaacg tatttttatt	660
gaattaaaaa ttacagttcg tgattgtaaa agttttccag gtggtgcaag tagttgtaaa	720
gaaacattta atttatatta tgcagaaaag gatttagatt atggtacaaa ttttcaaaaa	780
cgtttattta caaaaattga tacaattgca ccagatgaaa ttacagttag tagtgatttt	840
gaagcacgtc atgttaaaatt aaatgttgaa gaacgtagtg ttggtccatt aacacgtaaa	900
ggtttttatt tagcatttca agatattggt gcattgtgtt cattattaag ttttcgtgtt	960
tattataaaa aatgtccaga attattacaa ggttttagcac attttccaga aacaattgca	1020
ggtagtgtat caccaagttt agcaacagtt gcaggtagat gtgttgatca tgcagttgtt	1080
ccaccaggtg gtgaagaacc acgtatgcat tgtgcagttg atggtgaatg gttagttcca	1140
attggtcaat gtttatgtca agcaggttat gaaaaagttg aagatgcatg tcaagcatgt	1200
agtcacaggt tttttaaatt tgaagcaagt gaaagtccat gtttagaatg tccagaacat	1260
acattaccaa gtccagaagg tgcaacaagt tgtgaatgtg aagaaggttt ttttcgtgca	1320
ccacaagatc cagcaagtat gccatgtaca cgtccaccaa gtgcaccaca ttatttaaca	1380
gcagttggtg tgggtgcata agttgaatta cgttggacac caccacaaga tagtggtgtg	1440
cgtgaagata ttgtttatag tgttacatgt gaacaatgtt ggcagaaaag tggtagaatg	1500
ggtccatgtg aagcaaggtt tcgttatagt gaaccaccac atggtttaac acgtacaagt	1560
gttacagtta gtgatttaga accacatatg aattatacat ttacagttga agcacgtaat	1620
ggtgttagtg gtttagttac aagtcgtagt tttcgtacag caagtgttag tattaatcaa	1680
acagaaccac caaaagttcg tttagaaggt cgtagtacaa caagtttaag tgttagttgg	1740
agtattccac caccacaaca aagtcgtgtt tggaaaatag aagttacata tcgtaaaaaa	1800
ggtgatagta atagttataa ttttcgtcgt acagaaggtt ttagtgttac attagatgat	1860
ttagcaccag atacaacata tttagttcaa gttcaagcat taacacaaga aggtcaaggt	1920

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gcaggtagtc gtgttcacga atttcaaca gaacaaaaat taattagtga agaagattta 1980
tgagagctc 1989

<210> SEQ ID NO 13
<211> LENGTH: 581
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 13

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu
1 5 10 15
Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys
20 25 30
Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser
35 40 45
Pro Lys Thr Pro Ile Glu Lys Lys His Ala Asp Gly Ser Asp Tyr Lys
50 55 60
Asp Asp Asp Asp Lys Gln Gly Lys Glu Val Val Leu Leu Asp Phe Ala
65 70 75 80
Ala Ala Gly Gly Glu Leu Gly Trp Leu Thr His Pro Tyr Gly Lys Gly
85 90 95
Trp Asp Leu Met Gln Asn Ile Met Asn Asp Met Pro Ile Tyr Met Tyr
100 105 110
Ser Val Cys Asn Val Met Ser Gly Asp Gln Asp Asn Trp Leu Arg Thr
115 120 125
Asn Trp Val Tyr Arg Gly Glu Ala Glu Arg Ile Phe Ile Glu Leu Lys
130 135 140
Phe Thr Val Arg Asp Cys Asn Ser Phe Pro Gly Gly Ala Ser Ser Cys
145 150 155 160
Lys Glu Thr Phe Asn Leu Tyr Tyr Ala Glu Ser Asp Leu Asp Tyr Gly
165 170 175
Thr Asn Phe Gln Lys Arg Leu Phe Thr Lys Ile Asp Thr Ile Ala Pro
180 185 190
Asp Glu Ile Thr Val Ser Ser Asp Phe Glu Ala Arg His Val Lys Leu
195 200 205
Asn Val Glu Glu Arg Ser Val Gly Pro Leu Thr Arg Lys Gly Phe Tyr
210 215 220
Leu Ala Phe Gln Asp Ile Gly Ala Cys Val Ala Leu Leu Ser Val Arg
225 230 235 240
Val Tyr Tyr Lys Lys Cys Pro Glu Leu Leu Gln Gly Leu Ala His Phe
245 250 255
Pro Glu Thr Ile Ala Gly Ser Asp Ala Pro Ser Leu Ala Thr Val Ala
260 265 270
Gly Thr Cys Val Asp His Ala Val Val Pro Pro Gly Gly Glu Glu Pro
275 280 285
Arg Met His Cys Ala Val Asp Gly Glu Trp Leu Val Pro Ile Gly Gln
290 295 300
Cys Leu Cys Gln Ala Gly Tyr Glu Lys Val Glu Asp Ala Cys Gln Ala
305 310 315 320
Cys Ser Pro Gly Phe Phe Lys Phe Glu Ala Ser Glu Ser Pro Cys Leu
325 330 335

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Glu Cys Pro Glu His Thr Leu Pro Ser Pro Glu Gly Ala Thr Ser Cys
 340 345 350
 Glu Cys Glu Glu Gly Phe Phe Arg Ala Pro Gln Asp Pro Ala Ser Met
 355 360 365
 Pro Cys Thr Arg Pro Pro Ser Ala Pro His Tyr Leu Thr Ala Val Gly
 370 375 380
 Met Gly Ala Lys Val Glu Leu Arg Trp Thr Pro Pro Gln Asp Ser Gly
 385 390 395 400
 Gly Arg Glu Asp Ile Val Tyr Ser Val Thr Cys Glu Gln Cys Trp Pro
 405 410 415
 Glu Ser Gly Glu Cys Gly Pro Cys Glu Ala Ser Val Arg Tyr Ser Glu
 420 425 430
 Pro Pro His Gly Leu Thr Arg Thr Ser Val Thr Val Ser Asp Leu Glu
 435 440 445
 Pro His Met Asn Tyr Thr Phe Thr Val Glu Ala Arg Asn Gly Val Ser
 450 455 460
 Gly Leu Val Thr Ser Arg Ser Phe Arg Thr Ala Ser Val Ser Ile Asn
 465 470 475 480
 Gln Thr Glu Pro Pro Lys Val Arg Leu Glu Gly Arg Ser Thr Thr Ser
 485 490 495
 Leu Ser Val Ser Trp Ser Ile Pro Pro Pro Gln Gln Ser Arg Val Trp
 500 505 510
 Lys Tyr Glu Val Thr Tyr Arg Lys Lys Gly Asp Ser Asn Ser Tyr Asn
 515 520 525
 Val Arg Arg Thr Glu Gly Phe Ser Val Thr Leu Asp Asp Leu Ala Pro
 530 535 540
 Asp Thr Thr Tyr Leu Val Gln Val Gln Ala Leu Thr Gln Glu Gly Gln
 545 550 555 560
 Gly Ala Gly Ser Arg Val His Glu Phe Gln Thr Glu Gln Lys Leu Ile
 565 570 575
 Ser Glu Glu Asp Leu
 580

<210> SEQ ID NO 14
 <211> LENGTH: 1968
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette, encodes fusion protein

<400> SEQUENCE: 14

ggtacctcct ttgattagta ttttcctatc ttaaagttac ttttatgtgg aggcattaac 60
 atttgttaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata 120
 atattgcggt tcactcttag aagcgaattt cgccaatatt ataattatca aaagagaggg 180
 gtggcaaacg gtatttggca ttattagggt aaaaaatgta gaaggagagt gaaacccatg 240
 gcatacgaca gtcggtttga tgaatgggta cagaaactga aagaggaaag ctttcaaac 300
 aatacgtttg acgcgcgcaa atttattcaa ggagcgggga agattgcagg actttctctt 360
 ggattaacga ttgcccagtc ggttggggcc tttggatccg attataaaga tgatgatgat 420
 aaacaaggta aagaagttgt tttattagat tttgcagcag caggtggtga attaggttgg 480
 ttaacacatc catatggtaa aggttgggat ttaatgcaa atattatgaa tgatatgcca 540

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atttatatgt atagtgtttg taatgttatg agtgggtgatc aagataattg gttacgtaca    600
aattggggttt atcgtggtga agcagaacgt atttttattg aattaaaatt tacagttcgt    660
gattgtaata gttttccagg tgggtcaagt agttgtaaag aaacatttaa tttatattat    720
gcagaaagtg atttagatta tgggtacaaat tttcaaaaac gtttatttac aaaaattgat    780
acaattgcac cagatgaaat tacagttagt agtgattttg aagcacgtca tgtaaatta    840
aatgttgaag aacgtagtgt tgggtccatta acacgtaaag gtttttattt agcatttcaa    900
gatattgggtg catgtgttgc attattaagt gttcgtgttt attataaaaa atgtccagaa    960
ttattacaag gtttagcaca ttttccagaa acaattgcag gtagtgatgc accaagttta   1020
gcaacagttg caggtacatg tgttgatcat gcagttgttc caccagggtg tgaagaacca   1080
cgtatgcatt gtgcagttga tgggtgaatgg ttagttccaa ttggtcaatg tttatgtcaa   1140
gcagggttatg aaaaagttga agatgcatgt caagcatgta gtccagggtt ttttaaattt   1200
gaagcaagtg aaagtccatg tttagaatgt ccagaacata cattaccaag tccagaaggt   1260
gcaacaagtt gtgaatgtga agaaggtttt tttcgtgcac cacaagatcc agcaagtatg   1320
ccatgtacac gtccaccaag tgcaccacat tatttaacag cagttggtat ggggtgcaaaa   1380
gttgaattac gttggacacc accacaagat agtgggtggc gtgaagatat tgtttatagt   1440
gttacatgtg aacaatgttg gccagaaagt ggtgaatgtg gtccatgtga agcaagtgtt   1500
cgttatagtg aaccaccaca tgggttaaca cgtacaagtg ttacagttag tgatttagaa   1560
ccacatatga attatacatt tacagttgaa gcacgtaatg gtgttagtgg tttagttaca   1620
agtcgtagtt ttcgtacagc aagtgttagt attaatcaaa cagaaccacc aaaagttcgt   1680
ttagaaggtc gtagtacaac aagtttaagt gttagttgga gtattccacc accacaacaa   1740
agtcgtgttt ggaatatga agttacatat cgtaaaaaag gtgatagtaa tagttataat   1800
gttcgtcgta cagaaggttt tagtgttaca ttatgatgatt tagcaccaga tacaacatat   1860
ttagttcaag ttcaagcatt aacacaagaa ggtcaagggtg caggtagtcg tgttcatgaa   1920
tttcaaacag aacaaaaatt aattagtga gaaagatttat gagagctc                   1968

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<210> SEQ ID NO 15

<211> LENGTH: 574

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 15

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Met Ala Tyr Asp Ser Arg Phe Asp Glu Trp Val Gln Lys Leu Lys Glu
 1             5             10             15

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Glu Ser Phe Gln Asn Asn Thr Phe Asp Arg Arg Lys Phe Ile Gln Gly
 20             25             30

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Ala Gly Lys Ile Ala Gly Leu Ser Leu Gly Leu Thr Ile Ala Gln Ser
 35             40             45

```

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Val Gly Ala Phe Gly Ser Asp Tyr Lys Asp Asp Asp Asp Lys Gln Gly
 50             55             60

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Lys Glu Val Val Leu Leu Asp Phe Ala Ala Ala Gly Gly Glu Leu Gly
 65             70             75             80

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Trp Leu Thr His Pro Tyr Gly Lys Gly Trp Asp Leu Met Gln Asn Ile
 85             90             95

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Met	Asn	Asp	Met	Pro	Ile	Tyr	Met	Tyr	Ser	Val	Cys	Asn	Val	Met	Ser
	100							105					110		
Gly	Asp	Gln	Asp	Asn	Trp	Leu	Arg	Thr	Asn	Trp	Val	Tyr	Arg	Gly	Glu
	115						120					125			
Ala	Glu	Arg	Ile	Phe	Ile	Glu	Leu	Lys	Phe	Thr	Val	Arg	Asp	Cys	Asn
	130					135					140				
Ser	Phe	Pro	Gly	Gly	Ala	Ser	Ser	Cys	Lys	Glu	Thr	Phe	Asn	Leu	Tyr
	145				150					155				160	
Tyr	Ala	Glu	Ser	Asp	Leu	Asp	Tyr	Gly	Thr	Asn	Phe	Gln	Lys	Arg	Leu
			165					170						175	
Phe	Thr	Lys	Ile	Asp	Thr	Ile	Ala	Pro	Asp	Glu	Ile	Thr	Val	Ser	Ser
		180						185					190		
Asp	Phe	Glu	Ala	Arg	His	Val	Lys	Leu	Asn	Val	Glu	Glu	Arg	Ser	Val
		195					200					205			
Gly	Pro	Leu	Thr	Arg	Lys	Gly	Phe	Tyr	Leu	Ala	Phe	Gln	Asp	Ile	Gly
	210					215						220			
Ala	Cys	Val	Ala	Leu	Leu	Ser	Val	Arg	Val	Tyr	Tyr	Lys	Lys	Cys	Pro
	225				230					235					240
Glu	Leu	Leu	Gln	Gly	Leu	Ala	His	Phe	Pro	Glu	Thr	Ile	Ala	Gly	Ser
			245					250						255	
Asp	Ala	Pro	Ser	Leu	Ala	Thr	Val	Ala	Gly	Thr	Cys	Val	Asp	His	Ala
		260						265					270		
Val	Val	Pro	Pro	Gly	Gly	Glu	Glu	Pro	Arg	Met	His	Cys	Ala	Val	Asp
		275					280					285			
Gly	Glu	Trp	Leu	Val	Pro	Ile	Gly	Gln	Cys	Leu	Cys	Gln	Ala	Gly	Tyr
	290					295						300			
Glu	Lys	Val	Glu	Asp	Ala	Cys	Gln	Ala	Cys	Ser	Pro	Gly	Phe	Phe	Lys
	305				310					315					320
Phe	Glu	Ala	Ser	Glu	Ser	Pro	Cys	Leu	Glu	Cys	Pro	Glu	His	Thr	Leu
			325						330					335	
Pro	Ser	Pro	Glu	Gly	Ala	Thr	Ser	Cys	Glu	Cys	Glu	Glu	Gly	Phe	Phe
		340						345					350		
Arg	Ala	Pro	Gln	Asp	Pro	Ala	Ser	Met	Pro	Cys	Thr	Arg	Pro	Pro	Ser
		355					360					365			
Ala	Pro	His	Tyr	Leu	Thr	Ala	Val	Gly	Met	Gly	Ala	Lys	Val	Glu	Leu
		370				375					380				
Arg	Trp	Thr	Pro	Pro	Gln	Asp	Ser	Gly	Gly	Arg	Glu	Asp	Ile	Val	Tyr
	385				390					395					400
Ser	Val	Thr	Cys	Glu	Gln	Cys	Trp	Pro	Glu	Ser	Gly	Glu	Cys	Gly	Pro
			405						410					415	
Cys	Glu	Ala	Ser	Val	Arg	Tyr	Ser	Glu	Pro	Pro	His	Gly	Leu	Thr	Arg
		420						425					430		
Thr	Ser	Val	Thr	Val	Ser	Asp	Leu	Glu	Pro	His	Met	Asn	Tyr	Thr	Phe
		435					440					445			
Thr	Val	Glu	Ala	Arg	Asn	Gly	Val	Ser	Gly	Leu	Val	Thr	Ser	Arg	Ser
		450				455					460				
Phe	Arg	Thr	Ala	Ser	Val	Ser	Ile	Asn	Gln	Thr	Glu	Pro	Pro	Lys	Val
	465				470					475					480
Arg	Leu	Glu	Gly	Arg	Ser	Thr	Thr	Ser	Leu	Ser	Val	Ser	Trp	Ser	Ile
			485						490					495	

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Pro	Pro	Pro	Gln	Gln	Ser	Arg	Val	Trp	Lys	Tyr	Glu	Val	Thr	Tyr	Arg	
			500					505					510			
Lys	Lys	Gly	Asp	Ser	Asn	Ser	Tyr	Asn	Val	Arg	Arg	Thr	Glu	Gly	Phe	
		515					520					525				
Ser	Val	Thr	Leu	Asp	Asp	Leu	Ala	Pro	Asp	Thr	Thr	Tyr	Leu	Val	Gln	
	530					535					540					
Val	Gln	Ala	Leu	Thr	Gln	Glu	Gly	Gln	Gly	Ala	Gly	Ser	Arg	Val	His	
545					550				555					560		
Glu	Phe	Gln	Thr	Glu	Gln	Lys	Leu	Ile	Ser	Glu	Glu	Asp	Leu			
			565					570								

<210> SEQ ID NO 16
 <211> LENGTH: 1254
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens
 <400> SEQUENCE: 16

caccgcagga ggaagaacca gcgtgcccgc cagtccccgc aggacgttta cttctccaag	60
tcagaacaac tgaagcccct gaagacatac gtggaccccc acacatatga ggacccaac	120
caggctgtgt tgaagttcac taccgagatc catccatcct gtgtcactcg gcagaaggtg	180
atcggagcag gagagtttgg ggaggtgtac aagggcatgc tgaagacatc ctcggggaaag	240
aaggaggtgc cgttgcccat caagacgctg aaagccggct acacagagaa gcagcgagtg	300
gacttcctcg gcgaggcccg catcatgggc cagttcagcc accacaacat catccgccta	360
gagggcgctc tctccaaata caagcccatg atgatcatca ctgagtacat ggagaatggg	420
gccctggaca agttccttcg ggagaaggat ggcgagttca gcgtgctgca gctgggtggc	480
atgctgctgg gcacgcagc tggcatgaag tacctggcca acatgaacta tgtgcaccgt	540
gacctggctg cccgcaacat cctcgtcaac agcaacctgg tctgcaaggt gtctgacttt	600
ggcctgtccc gcgtgctgga ggacgacccc gaggccacct acaccaccag tggcggcaag	660
atccccatcc gctggaccgc cccggaggcc atttcctacc ggaagttcac ctctgccagc	720
gacgtgtgga gctttggcat tgtcatgtgg gaggtgatga cctatggcga gcggccctac	780
tgggagttgt ccaaccacga ggtgatgaaa gccatcaatg atggcttcgg gctccccaca	840
cccatggact gccctccgc catctaccag ctcatgatgc agtgctggca gcaggagcgt	900
gcccgcgcc ccaagttcgc tgacatcgtc agcatcctgg acaagctcat tcgtgccctt	960
gactccctca agaccctggc tgactttgac cccgcgtgt ctatccggct cccagcacg	1020
agcggctcgg agggggtgcc cttccgcacg gtgtccgagt ggctggagtc catcaagatg	1080
cagcagtata cggagcactt catggcggcc ggctacactg ccatcgagaa ggtggtgcag	1140
atgaccaacg acgacatcaa gaggattggg gtgcggctgc ccggccacca gaagcgcac	1200
gcctacagcc tgctgggact caaggaccag gtgaacactg tggggatccc catc	1254

<210> SEQ ID NO 17
 <211> LENGTH: 1254
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Codon-optimized sequence
 <400> SEQUENCE: 17

cacagacgta gaaaaaatca acgtgctcga caatccccag aagatgtgta tttttcgaaa	60
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agtgaacaat taaaaccatt aaaaacttat gttgatccgc atacgtacga agacccaaat 120
caagcagtat taaaatttac aacagaaata caccacaggt gtgttacaag acaaaaagtt 180
attggagcag gtgaattcgg agaggtatat aaaggtatgt taaaacatc atcaggtaaa 240
aaagaagttc cggttgcaat taaaacctta aaggcaggat atacagaaaa acagcgagtt 300
gattttttag gtgaagcagg aattatgggt caatttagcc atcataatat tattcgtttg 360
gaaggagtaa taagtaataa taaaccaatg atgattatta cagaatacat ggaaaacggt 420
gcttttagata aattttttacg tgaaaaggat ggtgaattta gtgttttaca attggttggt 480
atgttaagag gaattgctgc aggtatgaaa tatttagcta atatgaatta tgttcaccgt 540
gatttggcag caagaaatat cctagtcaat tccaatttag tatgtaaagt tagtgatttt 600
ggtttaagca gagtattaga agacgatcca gaggcaacct atacaacatc gggaggtaaa 660
attcctattc gttggacagc accagaagct atcagttacc gtaaatttac aagtgcacga 720
gacgtgtgga gttttggggt tgtaatgtgg gaagttatga catatggaga aagaccatat 780
tggaattaa gtaatcatga agttatgaaa gcaattaacg atggatttag attaccaact 840
ccgatggatt gtccatctgc catttatcaa ctaatgatgc aatgttggca acaagaaaga 900
gcacgacgtc caaaatttgc agatattggt agtattttag acaaatnaat tcgtgcacca 960
gatagtttaa aaacttttag agactttgat cctcgtgtta gtattcgatt accaagtacg 1020
tcaggttccg aaggagttcc atttcgcaca gtctccgaat ggttggaaac aattaaaatg 1080
caacaataca ccgaacactt tatggcagca ggttacacag caatcgaata agttgttcaa 1140
atgacaaaatg atgatatcaa acgtattgga gttagattac caggccacca gaaacgtatt 1200
gcatattctt tattaggttt aaaagatcaa gtaataaccg tgggaattcc aatt 1254

```

<210> SEQ ID NO 18

<211> LENGTH: 456

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

```

Val His Glu Phe Gln Thr Leu Ser Pro Glu Gly Ser Gly Asn Leu Ala
  1             5             10             15

```

```

Val Ile Gly Gly Val Ala Val Gly Val Val Leu Leu Leu Val Leu Ala
  20             25             30

```

```

Gly Val Gly Phe Phe Ile His Arg Arg Arg Lys Asn Gln Arg Ala Arg
  35             40             45

```

```

Gln Ser Pro Glu Asp Val Tyr Phe Ser Lys Ser Glu Gln Leu Lys Pro
  50             55             60

```

```

Leu Lys Thr Tyr Val Asp Pro His Thr Tyr Glu Asp Pro Asn Gln Ala
  65             70             75             80

```

```

Val Leu Lys Phe Thr Thr Glu Ile His Pro Ser Cys Val Thr Arg Gln
  85             90             95

```

```

Lys Val Ile Gly Ala Gly Glu Phe Gly Glu Val Tyr Lys Gly Met Leu
  100            105            110

```

```

Lys Thr Ser Ser Gly Lys Lys Glu Val Pro Val Ala Ile Lys Thr Leu
  115            120            125

```

```

Lys Ala Gly Tyr Thr Glu Lys Gln Arg Val Asp Phe Leu Gly Glu Ala
  130            135            140

```

-continued

Gly	Ile	Met	Gly	Gln	Phe	Ser	His	His	Asn	Ile	Ile	Arg	Leu	Glu	Gly
145				150					155					160	
Val	Ile	Ser	Lys	Tyr	Lys	Pro	Met	Met	Ile	Ile	Thr	Glu	Tyr	Met	Glu
			165						170					175	
Asn	Gly	Ala	Leu	Asp	Lys	Phe	Leu	Arg	Glu	Lys	Asp	Gly	Glu	Phe	Ser
			180					185					190		
Val	Leu	Gln	Leu	Val	Gly	Met	Leu	Arg	Gly	Ile	Ala	Ala	Gly	Met	Lys
		195					200					205			
Tyr	Leu	Ala	Asn	Met	Asn	Tyr	Val	His	Arg	Asp	Leu	Ala	Ala	Arg	Asn
	210					215					220				
Ile	Leu	Val	Asn	Ser	Asn	Leu	Val	Cys	Lys	Val	Ser	Asp	Phe	Gly	Leu
225					230					235					240
Ser	Arg	Val	Leu	Glu	Asp	Asp	Pro	Glu	Ala	Thr	Tyr	Thr	Thr	Ser	Gly
			245						250					255	
Gly	Lys	Ile	Pro	Ile	Arg	Trp	Thr	Ala	Pro	Glu	Ala	Ile	Ser	Tyr	Arg
			260					265						270	
Lys	Phe	Thr	Ser	Ala	Ser	Asp	Val	Trp	Ser	Phe	Gly	Ile	Val	Met	Trp
		275					280					285			
Glu	Val	Met	Thr	Tyr	Gly	Glu	Arg	Pro	Tyr	Trp	Glu	Leu	Ser	Asn	His
	290					295					300				
Glu	Val	Met	Lys	Ala	Ile	Asn	Asp	Gly	Phe	Arg	Leu	Pro	Thr	Pro	Met
305					310					315					320
Asp	Cys	Pro	Ser	Ala	Ile	Tyr	Gln	Leu	Met	Met	Gln	Cys	Trp	Gln	Gln
			325						330					335	
Glu	Arg	Ala	Arg	Arg	Pro	Lys	Phe	Ala	Asp	Ile	Val	Ser	Ile	Leu	Asp
		340					345						350		
Lys	Leu	Ile	Arg	Ala	Pro	Asp	Ser	Leu	Lys	Thr	Leu	Ala	Asp	Phe	Asp
	355					360						365			
Pro	Arg	Val	Ser	Ile	Arg	Leu	Pro	Ser	Thr	Ser	Gly	Ser	Glu	Gly	Val
	370				375						380				
Pro	Phe	Arg	Thr	Val	Ser	Glu	Trp	Leu	Glu	Ser	Ile	Lys	Met	Gln	Gln
385				390						395					400
Tyr	Thr	Glu	His	Phe	Met	Ala	Ala	Gly	Tyr	Thr	Ala	Ile	Glu	Lys	Val
			405						410					415	
Val	Gln	Met	Thr	Asn	Asp	Asp	Ile	Lys	Arg	Ile	Gly	Val	Arg	Leu	Pro
		420						425					430		
Gly	His	Gln	Lys	Arg	Ile	Ala	Tyr	Ser	Leu	Leu	Gly	Leu	Lys	Asp	Gln
	435						440					445			
Val	Asn	Thr	Val	Gly	Ile	Pro	Ile								
	450					455									

<210> SEQ ID NO 19

<211> LENGTH: 1437

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fusion protein coding sequence

<400> SEQUENCE: 19

atgaaaaaaa taatgctagt ttttattaca cttatattag ttagtctacc aattgcgcaa 60

caaaactgaag caaaggatgc atctgcattc aataaagaaa attcaatttc atccatggca 120

ccaccagcat ctccgcctgc aagtcctaag acgccaatcg aaaagaaaca cgcggatctc 180

-continued

```

gagcaccgca ggaggaagaa ccagcgtgcc cgccagtcce cggaggacgt ttactttctcc 240
aagtcagaac aactgaagcc cctgaagaca tacgtggacc cccacacata tgaggacccc 300
aaccaggctg tgttgaagtt cactaccgag atccatccat cctgtgtcac tcggcagaag 360
gtgatcggag caggagagtt tggggagggtg tacaagggca tgctgaagac atcctcgggg 420
aagaaggagg tgccggtggc catcaagacg ctgaaagccg gctacacaga gaagcagcga 480
gtggacttcc tcggcgaggc cggcatcatg ggccagtcca gccaccacaa catcatccgc 540
ctagagggcg tcactctcaa atacaagccc atgatgatca tcaactgagta catggagaat 600
ggggccctgg acaagttcct tcgggagaag gatggcgagt tcagcgtgct gcagctgggtg 660
ggcatgctgc ggggcatcgc agctggcatg aagtacctgg ccaacatgaa ctatgtgcac 720
cgtgacctgg ctgcccgcga catcctcgtc aacagcaacc tggctctgaa ggtgtctgac 780
tttgccctgt cccgcgtgct ggaggacgac cccgaggcca cctacaccac cagtggcggc 840
aagatcccca tccgtgggac cgccccggag gccatttcct accggaagtt cacctctgcc 900
agcgacgtgt ggagctttgg cattgtcatg tgggaggtga tgacctatgg cgagcggccc 960
tactgggagt tgtccaacca cgaggtgatg aaagccatca atgatggctt ccggctcccc 1020
acacccatgg actgcccctc cgccatctac cagctcatga tgcagtgtcg gcagcaggag 1080
cgtgcccgcc gccccaagtt cgctgacatc gtcagcatcc tggacaagct cattcgtgcc 1140
cctgactccc tcaagaccct ggctgacttt gacccccgcg tgtctatccg gctccccagc 1200
acgagcggct cggagggggt gcccttcgcg acggtgtccg agtggctgga gtccatcaag 1260
atgcagcagt atacggagca cttcatggcg gccggctaca ctgccatcga gaaggtggtg 1320
cagatgacca acgacgacat caagaggatt ggggtgcggc tgcccggcca ccagaagcgc 1380
atcgccatca gcctgctggg actcaaggac caggtgaaca ctgtggggat ccccatc 1437

```

<210> SEQ ID NO 20

<211> LENGTH: 479

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 20

```

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu
 1             5             10             15

```

```

Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys
 20             25             30

```

```

Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser
 35             40             45

```

```

Pro Lys Thr Pro Ile Glu Lys Lys His Ala Asp Leu Glu His Arg Arg
 50             55             60

```

```

Arg Lys Asn Gln Arg Ala Arg Gln Ser Pro Glu Asp Val Tyr Phe Ser
 65             70             75             80

```

```

Lys Ser Glu Gln Leu Lys Pro Leu Lys Thr Tyr Val Asp Pro His Thr
 85             90             95

```

```

Tyr Glu Asp Pro Asn Gln Ala Val Leu Lys Phe Thr Thr Glu Ile His
100             105             110

```

```

Pro Ser Cys Val Thr Arg Gln Lys Val Ile Gly Ala Gly Glu Phe Gly
115             120             125

```

-continued

Glu	Val	Tyr	Lys	Gly	Met	Leu	Lys	Thr	Ser	Ser	Gly	Lys	Lys	Glu	Val
130						135					140				
Pro	Val	Ala	Ile	Lys	Thr	Leu	Lys	Ala	Gly	Tyr	Thr	Glu	Lys	Gln	Arg
145				150					155					160	
Val	Asp	Phe	Leu	Gly	Glu	Ala	Gly	Ile	Met	Gly	Gln	Phe	Ser	His	His
			165					170						175	
Asn	Ile	Ile	Arg	Leu	Glu	Gly	Val	Ile	Ser	Lys	Tyr	Lys	Pro	Met	Met
			180					185					190		
Ile	Ile	Thr	Glu	Tyr	Met	Glu	Asn	Gly	Ala	Leu	Asp	Lys	Phe	Leu	Arg
		195					200					205			
Glu	Lys	Asp	Gly	Glu	Phe	Ser	Val	Leu	Gln	Leu	Val	Gly	Met	Leu	Arg
	210					215					220				
Gly	Ile	Ala	Ala	Gly	Met	Lys	Tyr	Leu	Ala	Asn	Met	Asn	Tyr	Val	His
225				230						235				240	
Arg	Asp	Leu	Ala	Ala	Arg	Asn	Ile	Leu	Val	Asn	Ser	Asn	Leu	Val	Cys
			245					250						255	
Lys	Val	Ser	Asp	Phe	Gly	Leu	Ser	Arg	Val	Leu	Glu	Asp	Asp	Pro	Glu
		260						265					270		
Ala	Thr	Tyr	Thr	Thr	Ser	Gly	Gly	Lys	Ile	Pro	Ile	Arg	Trp	Thr	Ala
		275					280					285			
Pro	Glu	Ala	Ile	Ser	Tyr	Arg	Lys	Phe	Thr	Ser	Ala	Ser	Asp	Val	Trp
	290					295					300				
Ser	Phe	Gly	Ile	Val	Met	Trp	Glu	Val	Met	Thr	Tyr	Gly	Glu	Arg	Pro
305				310						315				320	
Tyr	Trp	Glu	Leu	Ser	Asn	His	Glu	Val	Met	Lys	Ala	Ile	Asn	Asp	Gly
			325						330					335	
Phe	Arg	Leu	Pro	Thr	Pro	Met	Asp	Cys	Pro	Ser	Ala	Ile	Tyr	Gln	Leu
		340					345						350		
Met	Met	Gln	Cys	Trp	Gln	Gln	Glu	Arg	Ala	Arg	Arg	Pro	Lys	Phe	Ala
	355						360					365			
Asp	Ile	Val	Ser	Ile	Leu	Asp	Lys	Leu	Ile	Arg	Ala	Pro	Asp	Ser	Leu
	370				375							380			
Lys	Thr	Leu	Ala	Asp	Phe	Asp	Pro	Arg	Val	Ser	Ile	Arg	Leu	Pro	Ser
385				390						395				400	
Thr	Ser	Gly	Ser	Glu	Gly	Val	Pro	Phe	Arg	Thr	Val	Ser	Glu	Trp	Leu
			405						410					415	
Glu	Ser	Ile	Lys	Met	Gln	Gln	Tyr	Thr	Glu	His	Phe	Met	Ala	Ala	Gly
		420						425					430		
Tyr	Thr	Ala	Ile	Glu	Lys	Val	Val	Gln	Met	Thr	Asn	Asp	Asp	Ile	Lys
	435							440				445			
Arg	Ile	Gly	Val	Arg	Leu	Pro	Gly	His	Gln	Lys	Arg	Ile	Ala	Tyr	Ser
	450					455					460				
Leu	Leu	Gly	Leu	Lys	Asp	Gln	Val	Asn	Thr	Val	Gly	Ile	Pro	Ile	
465				470						475					

<210> SEQ ID NO 21

<211> LENGTH: 1737

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Expression cassette, encodes fusion protein

<400> SEQUENCE: 21

-continued

```

ggtaacctct ttgattagta tttcctatc ttaaagtac ttttatgtg aggcattaac    60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata    120
atattgcgtt tcattcttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtatttggca ttattagggt aaaaaatgta gaagagaggt gaaacccatg    240
aaaaaaataa tgctagtttt tattacactt atattagtta gtctaccaat tgcgcaacaa    300
actgaagcaa aggatgcacg tgcattcaat aaagaaaatt caatttcacg catggcacca    360
ccagcatctc cgctgcaagc tcctaagacg ccaatcgaag aaaaacacgc ggatggatcc    420
gattataaag atgatgatga taacacacaga cgtagaaaaa atcaacgtgc tcgacaatcc    480
ccagaagatg tgtatttttc gaaaagtga caattaaaac cattaataaac ttatgttgat    540
ccgcatacgt acgaagaccc aaatcaagca gtattaaaat ttacaacaga aatacaccca    600
agttgtgtta caagacaaaa agttattgga gcaggtgaat tcggagaggt atataaaggt    660
atgttaaaaa catcatcagg taaaaaagaa gttccggttg caattaaaac cttaaaggca    720
ggatatacag aaaaacagcg agttgatatt ttaggtgaag caggaattat gggtaattt    780
agccatcata atattattcg ttgggaagga gtaataagta aatataaacc aatgatgatt    840
attacagaat acatggaaaa cggtgcttta gataaatttt tacgtgaaaa ggatggtgaa    900
tttagtgatt tacaattggt tggtatgtta agaggaattg ctgcaggtat gaaatattta    960
gctaatatga attatgttca ccgtgatttg gcagcaagaa ataccctagt caattccaat   1020
ttagtatgta aagtttagta ttttggttta agcagagtat tagaagacga tccagaggca   1080
acctatacaa catcgggagg taaaattcct attcgttgga cagcaccaga agctatcagt   1140
taccgttaat ttacaagtcg atcacacgtg tggagttttg ggattgtaat gtgggaagtt   1200
atgacatatg gagaagagacc atattgggaa ttaagtaatc atgaagtat gaaagcaatt   1260
aacgatggat ttagattacc aectccgatg gattgtccat ctgccattta tcaactaatg   1320
atgcaatggt ggcaacaaga aagagcacga cgtccaaaat ttgcagatat tgtagtatt   1380
ttagacaaat taattcgtgc accagatagt ttaaaaactt tagcagactt tgatcctcgt   1440
gttagtattc gattaccaag tacgtcaggt tccgaaggag ttccatttcg cacagtctcc   1500
gaatgggttg aatcaattaa aatgcaacaa tacaccgaac actttatggc agcaggttac   1560
acagcaatcg aaaaagttgt tcaaatgaca aatgatgata ttaaacgtat tggagttaga   1620
ttaccaggcc accagaaacg tattgcatat tctttattag gtttaaaaga tcaagttaat   1680
accgtgggaa ttccaattga acaaaaatta atttccgaag aagacttata agagctc     1737

```

<210> SEQ ID NO 22

<211> LENGTH: 497

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 22

```

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu
 1             5             10             15

```

```

Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys
 20             25             30

```

```

Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser
 35             40             45

```

-continued

Pro	Lys	Thr	Pro	Ile	Glu	Lys	Lys	His	Ala	Asp	Gly	Ser	Asp	Tyr	Lys	50	55	60
Asp	Asp	Asp	Asp	Lys	His	Arg	Arg	Arg	Lys	Asn	Gln	Arg	Ala	Arg	Gln	65	70	75
Ser	Pro	Glu	Asp	Val	Tyr	Phe	Ser	Lys	Ser	Glu	Gln	Leu	Lys	Pro	Leu	85	90	95
Lys	Thr	Tyr	Val	Asp	Pro	His	Thr	Tyr	Glu	Asp	Pro	Asn	Gln	Ala	Val	100	105	110
Leu	Lys	Phe	Thr	Thr	Glu	Ile	His	Pro	Ser	Cys	Val	Thr	Arg	Gln	Lys	115	120	125
Val	Ile	Gly	Ala	Gly	Glu	Phe	Gly	Glu	Val	Tyr	Lys	Gly	Met	Leu	Lys	130	135	140
Thr	Ser	Ser	Gly	Lys	Lys	Glu	Val	Pro	Val	Ala	Ile	Lys	Thr	Leu	Lys	145	150	155
Ala	Gly	Tyr	Thr	Glu	Lys	Gln	Arg	Val	Asp	Phe	Leu	Gly	Glu	Ala	Gly	165	170	175
Ile	Met	Gly	Gln	Phe	Ser	His	His	Asn	Ile	Ile	Arg	Leu	Glu	Gly	Val	180	185	190
Ile	Ser	Lys	Tyr	Lys	Pro	Met	Met	Ile	Ile	Thr	Glu	Tyr	Met	Glu	Asn	195	200	205
Gly	Ala	Leu	Asp	Lys	Phe	Leu	Arg	Glu	Lys	Asp	Gly	Glu	Phe	Ser	Val	210	215	220
Leu	Gln	Leu	Val	Gly	Met	Leu	Arg	Gly	Ile	Ala	Ala	Gly	Met	Lys	Tyr	225	230	235
Leu	Ala	Asn	Met	Asn	Tyr	Val	His	Arg	Asp	Leu	Ala	Ala	Arg	Asn	Ile	245	250	255
Leu	Val	Asn	Ser	Asn	Leu	Val	Cys	Lys	Val	Ser	Asp	Phe	Gly	Leu	Ser	260	265	270
Arg	Val	Leu	Glu	Asp	Asp	Pro	Glu	Ala	Thr	Tyr	Thr	Thr	Ser	Gly	Gly	275	280	285
Lys	Ile	Pro	Ile	Arg	Trp	Thr	Ala	Pro	Glu	Ala	Ile	Ser	Tyr	Arg	Lys	290	295	300
Phe	Thr	Ser	Ala	Ser	Asp	Val	Trp	Ser	Phe	Gly	Ile	Val	Met	Trp	Glu	305	310	315
Val	Met	Thr	Tyr	Gly	Glu	Arg	Pro	Tyr	Trp	Glu	Leu	Ser	Asn	His	Glu	325	330	335
Val	Met	Lys	Ala	Ile	Asn	Asp	Gly	Phe	Arg	Leu	Pro	Thr	Pro	Met	Asp	340	345	350
Cys	Pro	Ser	Ala	Ile	Tyr	Gln	Leu	Met	Met	Gln	Cys	Trp	Gln	Gln	Glu	355	360	365
Arg	Ala	Arg	Arg	Pro	Lys	Phe	Ala	Asp	Ile	Val	Ser	Ile	Leu	Asp	Lys	370	375	380
Leu	Ile	Arg	Ala	Pro	Asp	Ser	Leu	Lys	Thr	Leu	Ala	Asp	Phe	Asp	Pro	385	390	395
Arg	Val	Ser	Ile	Arg	Leu	Pro	Ser	Thr	Ser	Gly	Ser	Glu	Gly	Val	Pro	405	410	415
Phe	Arg	Thr	Val	Ser	Glu	Trp	Leu	Glu	Ser	Ile	Lys	Met	Gln	Gln	Tyr	420	425	430
Thr	Glu	His	Phe	Met	Ala	Ala	Gly	Tyr	Thr	Ala	Ile	Glu	Lys	Val	Val	435	440	445

-continued

Gln Met Thr Asn Asp Asp Ile Lys Arg Ile Gly Val Arg Leu Pro Gly
450 455 460

His Gln Lys Arg Ile Ala Tyr Ser Leu Leu Gly Leu Lys Asp Gln Val
465 470 475 480

Asn Thr Val Gly Ile Pro Ile Glu Gln Lys Leu Ile Ser Glu Glu Asp
485 490 495

Leu

<210> SEQ ID NO 23

<211> LENGTH: 1737

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Expression cassette, encodes fusion protein

<400> SEQUENCE: 23

```

ggtagcctcct ttgattagta tttcctatc ttaaagttac ttttatgtgg aggcattaac    60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata    120
atattgcgtt tcatctttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtatttgcca ttattagggt aaaaatgta gaaggagagt gaaacccatg    240
aaaaaaatta tgttagtttt tattacatta attttagtta gtttaccat tgcacaacaa    300
acagaagcaa aagatgcaag tgcatttaat aaagaaaata gtattagtag tatggcacca    360
ccagcaagtc caccagcaag tccaaaaaca ccaattgaaa aaaaacatgc agatggatcc    420
gattataaag acgatgatga taaacacaga cgtagaaaaa atcaacgtgc tcgacaatcc    480
ccagaagatg tgtatttttc gaaaagtga caattaaaac cattaanaac ttatgttgat    540
ccgcatacgt acgaagacc aaatcaagca gtattaaaat ttacaacaga aatacaccca    600
agtttggtta caagacaaa agttattgga gcagggtgaat tcggagaggt atataaaggt    660
atgttaaaaa catcatcagg taaaaaagaa gttccggttg caattaaaac cttaaggca    720
ggatatacag aaaaacagcg agttgatttt ttagggtgaag caggaattat ggggtcaattt    780
agccatcata atattattcg ttgggaagga gtaataagta aatataaacc aatgatgatt    840
attacagaat acatggaaaa cggtgcttta gataaatttt tacgtgaaaa ggatggtgaa    900
tttagtggtt tacaattggt tggtagtga agaggaattg ctgcagggtat gaaatattta    960
gctaatatga attatgttca ccgtgatttg gcagcaagaa atatcctagt caattccaat   1020
ttagtatgta aagttagtga ttttggttta agcagagtat tagaagacga tccagaggca   1080
acctatacaa catcgggagg taaaattcct attcgttgga cagcaccaga agctatcagt   1140
taccgtaaat ttacaagtc atcagacgtg tggagttttg ggattgtaat gtgggaagtt   1200
atgacatatg gagaagacc atattgggaa ttaagtaatc atgaagttat gaaagcaatt   1260
aacgatggat ttagattacc aactccgatg gattgtccat ctgccattta tcaactaatg   1320
atgcaatggt ggcaacaaga aagagcacga cgtccaaaat ttgcagatat tgttagtatt   1380
ttagacaaat taattcgtgc accagatagt ttaaaaactt tagcagactt tgatcctcgt   1440
gttagtattc gattaccaag tacgtcaggt tccgaaggag ttccatttcg cacagtctcc   1500
gaatgggttg aatcaattaa atgcaacaa tacaccgaac actttatggc agcaggttac   1560
acagcaatcg aaaaagttgt tcaaatgaca aatgatgata ttaaacgtat tggagttaga   1620
ttaccaggcc accagaacg tattgcatat tctttattag gtttaaaaga tcaagttaat   1680

```

-continued

accgtgggaa ttccaattga acaaaaatta atttccgaag aagacttata agagctc 1737

<210> SEQ ID NO 24

<211> LENGTH: 497

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 24

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu
1 5 10 15Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys
20 25 30Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser
35 40 45Pro Lys Thr Pro Ile Glu Lys Lys His Ala Asp Gly Ser Asp Tyr Lys
50 55 60Asp Asp Asp Asp Lys His Arg Arg Arg Lys Asn Gln Arg Ala Arg Gln
65 70 75 80Ser Pro Glu Asp Val Tyr Phe Ser Lys Ser Glu Gln Leu Lys Pro Leu
85 90 95Lys Thr Tyr Val Asp Pro His Thr Tyr Glu Asp Pro Asn Gln Ala Val
100 105 110Leu Lys Phe Thr Thr Glu Ile His Pro Ser Cys Val Thr Arg Gln Lys
115 120 125Val Ile Gly Ala Gly Glu Phe Gly Glu Val Tyr Lys Gly Met Leu Lys
130 135 140Thr Ser Ser Gly Lys Lys Glu Val Pro Val Ala Ile Lys Thr Leu Lys
145 150 155 160Ala Gly Tyr Thr Glu Lys Gln Arg Val Asp Phe Leu Gly Glu Ala Gly
165 170 175Ile Met Gly Gln Phe Ser His His Asn Ile Ile Arg Leu Glu Gly Val
180 185 190Ile Ser Lys Tyr Lys Pro Met Met Ile Ile Thr Glu Tyr Met Glu Asn
195 200 205Gly Ala Leu Asp Lys Phe Leu Arg Glu Lys Asp Gly Glu Phe Ser Val
210 215 220Leu Gln Leu Val Gly Met Leu Arg Gly Ile Ala Ala Gly Met Lys Tyr
225 230 235 240Leu Ala Asn Met Asn Tyr Val His Arg Asp Leu Ala Ala Arg Asn Ile
245 250 255Leu Val Asn Ser Asn Leu Val Cys Lys Val Ser Asp Phe Gly Leu Ser
260 265 270Arg Val Leu Glu Asp Asp Pro Glu Ala Thr Tyr Thr Thr Ser Gly Gly
275 280 285Lys Ile Pro Ile Arg Trp Thr Ala Pro Glu Ala Ile Ser Tyr Arg Lys
290 295 300Phe Thr Ser Ala Ser Asp Val Trp Ser Phe Gly Ile Val Met Trp Glu
305 310 315 320Val Met Thr Tyr Gly Glu Arg Pro Tyr Trp Glu Leu Ser Asn His Glu
325 330 335

-continued

Val	Met	Lys	Ala	Ile	Asn	Asp	Gly	Phe	Arg	Leu	Pro	Thr	Pro	Met	Asp
			340					345					350		
Cys	Pro	Ser	Ala	Ile	Tyr	Gln	Leu	Met	Met	Gln	Cys	Trp	Gln	Gln	Glu
		355					360					365			
Arg	Ala	Arg	Arg	Pro	Lys	Phe	Ala	Asp	Ile	Val	Ser	Ile	Leu	Asp	Lys
	370					375					380				
Leu	Ile	Arg	Ala	Pro	Asp	Ser	Leu	Lys	Thr	Leu	Ala	Asp	Phe	Asp	Pro
385					390					395					400
Arg	Val	Ser	Ile	Arg	Leu	Pro	Ser	Thr	Ser	Gly	Ser	Glu	Gly	Val	Pro
				405					410					415	
Phe	Arg	Thr	Val	Ser	Glu	Trp	Leu	Glu	Ser	Ile	Lys	Met	Gln	Gln	Tyr
			420					425					430		
Thr	Glu	His	Phe	Met	Ala	Ala	Gly	Tyr	Thr	Ala	Ile	Glu	Lys	Val	Val
		435					440					445			
Gln	Met	Thr	Asn	Asp	Asp	Ile	Lys	Arg	Ile	Gly	Val	Arg	Leu	Pro	Gly
	450					455					460				
His	Gln	Lys	Arg	Ile	Ala	Tyr	Ser	Leu	Leu	Gly	Leu	Lys	Asp	Gln	Val
465					470					475					480
Asn	Thr	Val	Gly	Ile	Pro	Ile	Glu	Gln	Lys	Leu	Ile	Ser	Glu	Glu	Asp
				485					490					495	

Leu

<210> SEQ ID NO 25

<211> LENGTH: 1716

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Expression cassette, encodes fusion protein

<400> SEQUENCE: 25

```

ggtagcctcct ttgattagta tattcctatc ttaaagttac ttttatgtgg aggcattaac    60
atttggttaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata    120
atattgcggtt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtattttggca ttattaggtt aaaaaatgta gaaggagagt gaaacccatg    240
gcatacgaca gtcgttttga tgaatgggta cagaaactga aagaggaaag ctttcaaac    300
aatacgtttg accgccgcaa atttattcaa ggagcgggga agattgcagg actttctctt    360
ggattaacga ttgcccgatc ggttggggcc tttggatccg attataaaga tgatgatgat    420
aaacacagac gtagaaaaaa tcaacgtgct cgacaatccc cagaagatgt gtatttttcg    480
aaaagtgaac aattaaaacc attaaaaact tatgttgatc cgcatacgta cgaagacca    540
aatcaagcag tattaaaatt tacaacagaa atacacccaa gttgtgttac aagacaaaa    600
gttattggag caggtgaatt cggagaggta tataaaggta tgttaaaac atcatcaggt    660
aaaaaagaag ttccggttgc aattaaaacc ttaaaggcag gatatacaga aaaacagcga    720
gttgattttt taggtgaagc aggaattatg ggtcaattta gccatcataa tattattcgt    780
ttggaaggag taataagtaa atataaacca atgatgatta ttacagaata catggaaaac    840
ggtgcttttag ataaattttt acgtgaaaag gatggtgaat ttagtgtttt acaattgggt    900
ggtatgttaa gaggaattgc tgcaggtatg aaatatttag ctaatatgaa ttatgttcac    960
cgtgatttgg cagcaagaaa taccctagtc aattccaatt tagtatgtaa agttagtgat    1020

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-continued

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tttggtttaa gcagagtatt agaagacgat ccagaggcaa cctatacaac atcgggaggt 1080
aaaattccta ttcgttgac agcaccagaa gctatcagtt accgtaaatt tacaagtga 1140
tcagacgtgt ggagtttttg gattgtaatg tgggaagtta tgacatatgg agaaagacca 1200
tattgggaat taagtaatac tgaagttatg aaagcaatta acgatggatt tagattacca 1260
actccgatgg attgtccatc tgccatttat caactaatga tgcaatgttg gcaacaagaa 1320
agagcacgac gtccaaaatt tgcagatatt gttagtatgt tagacaaaatt aattcgtgca 1380
ccagatagtt taaaaacttt agcagacttt gatcctcgtg ttagtattcg attaccaagt 1440
acgtcagggt ccgaaggagt tccatttcgc acagtctcgc aatgggttga atcaattaaa 1500
atgcaacaat acaccgaaca ctttatggca gcaggttaca cagcaatcga aaaagttggt 1560
caaatgacaa atgatgatat taaacgtatt ggagttagat taccaggcca ccagaacgt 1620
attgcatatt ctttattagg tttaaaagat caagttaata ccgtgggaat tccaattgaa 1680
caaaaattaa tttccgaaga agacttataa gagctc 1716

```

<210> SEQ ID NO 26

<211> LENGTH: 490

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fusion protein

<400> SEQUENCE: 26

```

Met Ala Tyr Asp Ser Arg Phe Asp Glu Trp Val Gln Lys Leu Lys Glu
 1           5           10           15
Glu Ser Phe Gln Asn Asn Thr Phe Asp Arg Arg Lys Phe Ile Gln Gly
 20           25           30
Ala Gly Lys Ile Ala Gly Leu Ser Leu Gly Leu Thr Ile Ala Gln Ser
 35           40           45
Val Gly Ala Phe Gly Ser Asp Tyr Lys Asp Asp Asp Lys His Arg
 50           55           60
Arg Arg Lys Asn Gln Arg Ala Arg Gln Ser Pro Glu Asp Val Tyr Phe
 65           70           75           80
Ser Lys Ser Glu Gln Leu Lys Pro Leu Lys Thr Tyr Val Asp Pro His
 85           90           95
Thr Tyr Glu Asp Pro Asn Gln Ala Val Leu Lys Phe Thr Thr Glu Ile
100           105           110
His Pro Ser Cys Val Thr Arg Gln Lys Val Ile Gly Ala Gly Glu Phe
115           120           125
Gly Glu Val Tyr Lys Gly Met Leu Lys Thr Ser Ser Gly Lys Lys Glu
130           135           140
Val Pro Val Ala Ile Lys Thr Leu Lys Ala Gly Tyr Thr Glu Lys Gln
145           150           155           160
Arg Val Asp Phe Leu Gly Glu Ala Gly Ile Met Gly Gln Phe Ser His
165           170           175
His Asn Ile Ile Arg Leu Glu Gly Val Ile Ser Lys Tyr Lys Pro Met
180           185           190
Met Ile Ile Thr Glu Tyr Met Glu Asn Gly Ala Leu Asp Lys Phe Leu
195           200           205
Arg Glu Lys Asp Gly Glu Phe Ser Val Leu Gln Leu Val Gly Met Leu
210           215           220

```

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Arg Gly Ile Ala Ala Gly Met Lys Tyr Leu Ala Asn Met Asn Tyr Val	
225 230 235 240	
His Arg Asp Leu Ala Ala Arg Asn Ile Leu Val Asn Ser Asn Leu Val	
245 250 255	
Cys Lys Val Ser Asp Phe Gly Leu Ser Arg Val Leu Glu Asp Asp Pro	
260 265 270	
Glu Ala Thr Tyr Thr Thr Ser Gly Gly Lys Ile Pro Ile Arg Trp Thr	
275 280 285	
Ala Pro Glu Ala Ile Ser Tyr Arg Lys Phe Thr Ser Ala Ser Asp Val	
290 295 300	
Trp Ser Phe Gly Ile Val Met Trp Glu Val Met Thr Tyr Gly Glu Arg	
305 310 315 320	
Pro Tyr Trp Glu Leu Ser Asn His Glu Val Met Lys Ala Ile Asn Asp	
325 330 335	
Gly Phe Arg Leu Pro Thr Pro Met Asp Cys Pro Ser Ala Ile Tyr Gln	
340 345 350	
Leu Met Met Gln Cys Trp Gln Gln Glu Arg Ala Arg Arg Pro Lys Phe	
355 360 365	
Ala Asp Ile Val Ser Ile Leu Asp Lys Leu Ile Arg Ala Pro Asp Ser	
370 375 380	
Leu Lys Thr Leu Ala Asp Phe Asp Pro Arg Val Ser Ile Arg Leu Pro	
385 390 395 400	
Ser Thr Ser Gly Ser Glu Gly Val Pro Phe Arg Thr Val Ser Glu Trp	
405 410 415	
Leu Glu Ser Ile Lys Met Gln Gln Tyr Thr Glu His Phe Met Ala Ala	
420 425 430	
Gly Tyr Thr Ala Ile Glu Lys Val Val Gln Met Thr Asn Asp Asp Ile	
435 440 445	
Lys Arg Ile Gly Val Arg Leu Pro Gly His Gln Lys Arg Ile Ala Tyr	
450 455 460	
Ser Leu Leu Gly Leu Lys Asp Gln Val Asn Thr Val Gly Ile Pro Ile	
465 470 475 480	
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu	
485 490	

<210> SEQ ID NO 27

<211> LENGTH: 966

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Expression cassette, encodes fusion protein

<400> SEQUENCE: 27

ggtacctcct ttgattagta tttcctatc ttaaagttac ttttatgtgg aggcattaac	60
atttgttaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata	120
atattgcgtt tcattcttag aagcgaattt cgccaatatt ataattatca aaagagaggg	180
gtggcaaacg gtatttgcca ttattagggt aaaaaatgta gaaggagagt gaaacccatg	240
aaaaaaatta tgtagtttt tattacatta attttagtta gtttaccat tgcacaacaa	300
acagaagcaa aagatgcaag tgcatttaaat aaagaaaata gtattagtag tatggcacca	360
ccagcaagtc caccagcaag tccaaaaaca ccaattgaaa aaaaacatgc agatggatcc	420
caagcagaag gtcgcggaac aggaggaagt acaggagatg cagacggacc aggaggacca	480

-continued

```

ggaataccag acggaccagg aggaaatgca ggaggcccag gcgaagcagg cgcaacagga 540
ggaagaggac caagaggagc aggagcagca cgagcatcag gaccaggagg cgagcacca 600
agaggaccac atggcggagc ggcaagcgga ttaaattgat gttgtagatg tggagcacgc 660
ggaccagaat caagactttt agaattttat ttagccatgc catttgcaac cccaatggaa 720
gcagaattag cacgaagatc attagcaca gatgcccac cattaccagt accaggagtt 780
ttattaaaag agtttacagt atcaggcaat attttaaca tacgtttaac agcagcagac 840
catcgtcaat tacaactatc tatcagttca tgtttacaac aattatcctt attaatgtgg 900
attacacaat gttttttacc agttttttta gcacaaccac catcaggaca aagaagataa 960
gagctc

```

```

<210> SEQ ID NO 28
<211> LENGTH: 240
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Fusion protein

```

```

<400> SEQUENCE: 28

```

```

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu
 1             5             10            15
Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys
 20            25            30
Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser
 35            40            45
Pro Lys Thr Pro Ile Glu Lys Lys His Ala Asp Gly Ser Gln Ala Glu
 50            55            60
Gly Arg Gly Thr Gly Gly Ser Thr Gly Asp Ala Asp Gly Pro Gly Gly
 65            70            75            80
Pro Gly Ile Pro Asp Gly Pro Gly Gly Asn Ala Gly Gly Pro Gly Glu
 85            90            95
Ala Gly Ala Thr Gly Gly Arg Gly Pro Arg Gly Ala Gly Ala Ala Arg
100            105            110
Ala Ser Gly Pro Gly Gly Gly Ala Pro Arg Gly Pro His Gly Gly Ala
115            120            125
Ala Ser Gly Leu Asn Gly Cys Cys Arg Cys Gly Ala Arg Gly Pro Glu
130            135            140
Ser Arg Leu Leu Glu Phe Tyr Leu Ala Met Pro Phe Ala Thr Pro Met
145            150            155            160
Glu Ala Glu Leu Ala Arg Arg Ser Leu Ala Gln Asp Ala Pro Pro Leu
165            170            175
Pro Val Pro Gly Val Leu Leu Lys Glu Phe Thr Val Ser Gly Asn Ile
180            185            190
Leu Thr Ile Arg Leu Thr Ala Ala Asp His Arg Gln Leu Gln Leu Ser
195            200            205
Ile Ser Ser Cys Leu Gln Gln Leu Ser Leu Leu Met Trp Ile Thr Gln
210            215            220
Cys Phe Leu Pro Val Phe Leu Ala Gln Pro Pro Ser Gly Gln Arg Arg
225            230            235            240

```

```

<210> SEQ ID NO 29

```

-continued

<211> LENGTH: 330
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Partial expression cassette comprising codon-optimized sequence

<400> SEQUENCE: 29

```
ggtacctcct ttgattagta tattcctatc ttaaagttac ttttatgtgg aggcattaac    60
atttgtaaat gacgtcaaaa gtagtagcaag actagaataa agctataaag caagcatata    120
atattgcggt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtatttggca ttattaggtt aaaaaatgta gaaggagagt gaaacccatg    240
aaaaaaaaaa ttattagtgc aattttaatg agtacagtta ttttaagtgc agcagcacca    300
ttaagtgggt tttatgcaga tacaggatcc                                     330
```

<210> SEQ ID NO 30
<211> LENGTH: 330
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Partial expression cassette

<400> SEQUENCE: 30

```
ggtacctcct ttgattagta tattcctatc ttaaagttac ttttatgtgg aggcattaac    60
atttgtaaat gacgtcaaaa gtagtagcaag actagaataa agctataaag caagcatata    120
atattgcggt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtatttggca ttattaggtt aaaaaatgta gaaggagagt gaaacccatg    240
aatatgaaaa aagcaactat cgcggttaca gctgggattg cggtaacagc atttgcgtgcg    300
ccaacaatcg catccgcaag cactggatcc                                     330
```

<210> SEQ ID NO 31
<211> LENGTH: 330
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Partial expression cassette comprising codon-optimized sequence

<400> SEQUENCE: 31

```
ggtacctcct ttgattagta tattcctatc ttaaagttac ttttatgtgg aggcattaac    60
atttgtaaat gacgtcaaaa gtagtagcaag actagaataa agctataaag caagcatata    120
atattgcggt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtatttggca ttattaggtt aaaaaatgta gaaggagagt gaaacccatg    240
aatatgaaaa aagcaacaat tgcagcaaca gcaggatttg cagttacagc atttcagca    300
ccaacaattg caagtgcagg tacaggatcc                                     330
```

<210> SEQ ID NO 32
<211> LENGTH: 1702
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: hlyP-p60 gene fragment

<400> SEQUENCE: 32

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ggtacctcct ttgattagta ttttcctatc ttaaagttac ttttatgtgg aggcattaac	60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata	120
atattgcgtt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg	180
gtggcaaacg gtatttggca ttattagggt aaaaaatgta gaaggagagt gaaacccatg	240
aatatgaaaa aagcaactat cgcggctaca gctgggattg cggtaacagc atttgctgcg	300
ccaacaatcg catccgcaag cactgtagta gtcgaagctg gtgatactct ttggggatc	360
gcacaaagta aagggaactac tgttgacgca attaaaaaag caaacaattt aacaacagat	420
aaaatcgtac cagggtcaaaa attacaagta aataatgagg ttgctgctgc tgaaaaaca	480
gagaaatctg ttacgcaaac ttggttaaac gtccgtagtg gcgctgggtg tgataacagt	540
attattacgt ccatcaaaag tggaacaaaa gtaactgttg aaacaaccga atctaacggc	600
tggcacaaaa ttacttacaa cgatggaaaa actggtttcg ttaacggtaa atacttaact	660
gacaaagcag taagcactcc agttgcacca acacaagaag tgaaaaaaga aactactact	720
caacaagctg cacctgctgc agaaacaaaa actgaagtaa acaaaactac acaagcaact	780
acacctgcgc ctaaagtagc agaaacgaaa gaaactccag tagtagatca aaatgctact	840
acacacgctg ttaaaagcgg tgacactatt tgggctttat ccgtaaaata cgggtgttct	900
gttcaagaca ttatgtcatg gaataattta tcttcttctt ctatttatgt aggtcaaaag	960
cttgctatta aacaaactgc taacacagct actccaaaag cagaagtga aacggaagct	1020
ccagcagctg aaaaacaagc agctccagta gttaaagaaa atactaacac aaatactgct	1080
actacagaga aaaaagaaac agcaacgcaa caacaacag cacctaaagc accaacagaa	1140
gctgcaaac cagctcctgc accatctaca aacacaaatg ctaataaaac aaatacaaat	1200
acaaatacaa atacaaatc aaacaatact aatacaata caccatctaa aaatactaat	1260
acaaactcaa atactaatc gaatacaaac tcaaatcga atgctaatac aggttcttcc	1320
aaacaataca gcaattcaag tgcaagtgtc attattgctg aagctcaaaa acaccttgga	1380
aaagcttatt catgggggtg taacggacca actacatttg attgctctgg ttacactaaa	1440
tatgtatttg ctaaagcggg aatctccctt ccacgtactt ctggcgcaac atacgctagc	1500
actacaagaa tctctgaatc tcaagcaaaa cctgggtgatt tagtattctt tgactatggt	1560
agcgggaatt ctcacgttgg tatctacggt ggtaatggtc aaatgattaa cgcgcaagac	1620
aatggcggtt aatacgataa catccacggc tctggctggg gtaaatatct agttggcttc	1680
ggtcgcgtat aattaaggat cc	1702

<210> SEQ ID NO 33

<211> LENGTH: 9808

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: pAM401-MCS plasmid

<400> SEQUENCE: 33

ctttaaacgt ggatcatttt ctttaaattt atgctgacga ctttgaatt tgcctttttt	60
cttagcaatt tcgattcctt gtgcctgacg ttccttaatt tttttctggt ctgattctgc	120
ttgatacttg tacaattcaa tgacaaggct attaatcaaa cgccttaaat tttcatcttc	180
aataccattc attgagggtg aatttaagac ttccagggtt gcccccttaa tttgaatttg	240

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attcatcaat tctgttaatt ctttattatt tcgtcctaatt cgaatcaatt cagtaacaat	300
aacaatatcc ccttcacgaa tatagttaag catagcttgt aattgtgggc gtccgaccga	360
ttgaccgctt aatttgtctg aaaagacctt agaaacgccc tgtaacgctt gtaattgccg	420
atctaagttc tgttctttgc tactgacacg tgcataacca attttagcca ttttcaacca	480
acctctaaaa ttctctcggt tgcaataacc aatcagcaat atctactttt tcaatttcaa	540
attgcttatac agaaattgtc ttttcgtaag cgataaaatc ttgcgcataat tgttgctcat	600
taaaaatagc caccacttcg tcatttttcta aaactcgata aataaatttt ttcattttac	660
tcctcctatt atgcccactt taaatgacct attcaccaag tcaattatac tgctaaaaatc	720
atattaggac aatataggat actctattga cctataaatg atagcaactt aaaagatcaa	780
gtgttcgctt cgctctcact gccctcgcac gtttttagtag ctttccctc acttcgttca	840
gtccaagcca actaaaagtt ttccgggtac tctctccttc tccccctaata aattaattaa	900
aatcttactc tgtatatctt tgctaatacat tcaactaaca gcaaaagaaa acaaacacgt	960
atcatagata taaatgtaat ggcatagtc gggttttatt ttcagcctgt atcgtagcta	1020
aacaaatcga gtgtgtgggc cgttttgggg cgttctgccca atttgtttag agtttcttga	1080
ataaatgtac gttctaaatt aaacgaagct gtcagcgctt ttatataagct ttctcggtct	1140
tcctttttta atttaagat cgatagcaac aatgatttaa cactagcaag ttgaatgcca	1200
ccatttcttc ctggttttaatt cttaaagaaa atttctgat tcgccttcag taccttcagc	1260
aatttatcta atgtccgttc aggaatgcct agcactcttc taactctttt ttggtcgtc	1320
gctaataaag gcttgatata atcgcttttt tcgctaatat aagccattaa atcttcttct	1380
cattctgaca aatgaacacg ttgacgttcg cttctttttt tcttgaattt aaaccaccct	1440
tgacggacaa ataaatcttt actggttaaa tcacttgata cccaagcttt gcaaagaatg	1500
gtaatgtatt ccctattagc cccttgatag ttttctgaat aggcacttct aacaattttg	1560
attacttctt ttctctctaa ggggtgatct aatcgattat taaactcaaa catattatat	1620
tcgcacgttt cgattgaata gcctgaacta aagtaggcta aagagagggg aaacataacg	1680
ctattgcgcc ctactaaacc cttttctcct gaaaatttcg ttctgtgcaa taagagatta	1740
aaccagggtt catctacttg tttttgcct tctgtaccgc ttaaaaccgt tagacttgaa	1800
cgagtaaaag ccttattatc tgtttgtttg aaagaccaat cttgccattc ttgaaagaa	1860
taacggtaat tgggatcaaa aaattctaca ttgtccgttc ttggtatacg agcaatccca	1920
aaatgattgc acgttagatc aactggcaaa gactttccaa aatattctcg gatattttgc	1980
gagattattt tggctgcttt gacagattta aattctgatt ttgaagtac atagactggc	2040
gtttctaaaa caaaatatgc ttgataacct ttatcagatt tgataattaa cgtaggcata	2100
aaacctaaat caatagctgt tgtaaaata tcgcttgctg aaatagtttc ttttccgtg	2160
tgaatatcaa aatcaataaa gaaggatttg atttgtctta aattgttttc agaattgcct	2220
ttagtgtatg aacggttttc gcttgcatc gtaccataac gataaacgtt tgggtgccaa	2280
tcgctaagtg tatcttgatt ttctgtgaat gcttcttcgg aagtcagaac aacgccacgt	2340
ccgccaatca tgcctttttt tgagcgatac gcaaaaatag cccctttact ttacctggc	2400
ttggtagtga ttgagcgaat ttactattt ttaaatgtgt actttaaaaa gccgtcatga	2460
agcacagttt ctacaacaaa agggatatto attcagctgt tctcctttct tacgaaaatt	2520

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aattagttag aagctacgat caaagttgaa tcacaacaaa aaaggcaatc aactaagttt	2580
ttcttaattg attgcctggt atcttcttaa agacttgaaa tccctctaaa aaccgatat	2640
aatgggttta cagatattta agtatctgat taataaagta attaaatact ttaccaaatt	2700
ttgggtctcg acttctttta ttgattgggtg gtaatcaatt aaggctcgca gttaaaattt	2760
ctcaggcttt aactggctcg ggctcttttt ttgtattctt tattcagttc gttgtttcgt	2820
tatatctagt atatcgcttt ttaaaaaaat aagcaatgat ttcgtgcatt attcacacga	2880
aatcattgct tttttcttct tccatttcta actccaatgt tacttgttct gtttctgggt	2940
ctggttctgt tggctcattt gggattaaat ccactactag cgttgagtta gttccgtctc	3000
taatagccgg ttaagtaata gccggttaag tggtcaaaact ttgggaaaat ctcaaccgc	3060
attaagtttt gatgccatga caatcgttgg aaatttgaac aaaactaatg ctaaaaagct	3120
atctgacttt atgagtgtag agccacaaat acgactttgg gatatacttc aaacaaagtt	3180
taaagctaag gcacttcaag aaaaagttta tatcgaatat gacaaagtaa aagcagatac	3240
ttgggtaga cgtaatatgc gtgttgaatt taatcccaat aaactcacac atgaagaaat	3300
gatttggtta aaacaaaata ttatcgacta catggaagat gacggtttta caagattaga	3360
cttagctttt gattttgaag atgatttgag cgattactat gcaatgactg ataaagcagt	3420
taagaaact gttttttatg gtcgtaatgg caagccagaa acaaaatatt ttggtgtccg	3480
tgatagtgat agatttatta gaatttataa taaaaacaa gaacgtaag ataacgcaga	3540
tgttgaagtt gtgtttgaac atttatggcg tgtagaagtt gaattaaaa gagatatggt	3600
tgattactgg aatgattggt ttaatgattt acacatcttt gaaacctgag tgggctactt	3660
tagaaaaaat taatgagcaa gctatgggtt atactttggt gcatgaagaa agtatgtggg	3720
gaaagctaag taagaatact aagactaaat ttaaaaaatt gattagagaa atatctccaa	3780
ttgatttaac ggaattaatg aaatcgactt taaagcgaa cgaaaaaaa ttgcaaaagc	3840
agattgattt ttggcaacgt gaatttaggt ttggaagta aaataagttt tatttgataa	3900
aaattgctaa ttcagtataa ttaatattha cgaggtgaca taacgtatga aaaaatcaga	3960
ggattattcc tcctaataat aaaaatttaa aatttaggag gaagtatat atgactttta	4020
atattattga attagaaaa ttggtagaaa aagaatattt tgaacactat ttaaatcagc	4080
aaactactta tagcattact aaagaaattg atattacttt gtttaaagat atgataaaaa	4140
agaaaggata tgaattttat ccctctttaa tttatgcaat tatggaagtt gtaataaaaa	4200
ataaagtgtt tagaacagga attaatagtg agaataaatt aggttattgg gataagttaa	4260
atcctttgta tacagttttt aataagcaaa ctgaaaaaatt tactaacatt tggactgaat	4320
ctgataaaaa ctctatttct ttttataata attataaaaa tgacttgctt gaatataaag	4380
ataaagaaga aatgtttcct aaaaaaccga tacctgaaaa caccataccg atttcaatga	4440
ttccttggtt tgattttagt tcatttaatt taaatattgg taacaatagc agctttttat	4500
tgcctattat tacgataggt aaattttata gtgagaataa taaaatttat ataccagttg	4560
ctctgcaact tcactattct gtatgtgatg gttaccatgc ttcactattt atgaatgaat	4620
ttcaagatat aattcatagg gtatagattt ggatttagtt tttagatttt gaaagtgaat	4680
ttaattttat acacgtaagt gatcataaaa tttatgaacg tataacaacc acattttttg	4740
gttgcttggt gttttgattt tgaatttggt tttgaactta tggactgatt tattcagtc	4800

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atTTTTgtg cttgcacaaa aactagcctc gcagagcaca cgcattaatg acttatgaaa	4860
cgtagtaaat aagtctagtg tgttatactt tacttggaag atgcaccgaa taaaaaatat	4920
tgaagaacaa ctacgaaaaag attttaaaga gttattttat ttaagtctt tataacatga	4980
gtgaagcgaa tttttaaatt tcgatagaaa tttttacatc aaaaagcccc ctgtcaaaat	5040
tgacgaagg ggttttttgg cgcacgcttt tcgtagaaa tatacaagat tgaatatcgt	5100
gtataagtgc gccctttgtt ttgaacttag cacgttacat caatttttta aaatgatgta	5160
taagtgcgcc cttttaaatt ttgagtgtt atattttttg agttagaaaa agggattggg	5220
aaaatttccc aaaataattt aaaaaataag caaaaatttt cgatagagaa tgtgtattt	5280
tttgtcaag gtgtatacct tgactgtgtc tgctgttaca ttaagtttat ttttaagtta	5340
ttaaaaaaga aatagctttt aaagtgttgc tcgctgtcgc tttataaagc tgattgactt	5400
ttgattgcaa actacttaaa gaaaacaaac tcggactatt cgttttcttc tctttggtt	5460
gaacatcagc aattatcccc tcttgattgc ctatttttagc ttgtttagaa gaaacaaaag	5520
ctaaaagtc ctcttgggtt ttaaaacgct gtgtggggct tagaacgccc ttaaacgacc	5580
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gcggtccagt gatcgaagtt aggcgtgtaa gagccgcgag cgatccttga agctgtccct	6780
gatggtcgtc atctacctgc ctggacagca tggcctgcaa cgcgggcac ccgatgccgc	6840
cggagcgag aagaatcata atggggaagg ccattccagc tcgctcgca atacgactca	6900
ctataggcg aattgggtac cgggcccccc ctcgaggtcg acggtatcga taagcttgat	6960
atcgaattcc tgcagcccg gggtaccact agttctagag cggccgccac cgcggtggag	7020
ctccagcttt tgttcccttt agtgagggtt aatgctagaa atattttatc tgattaataa	7080

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gatgatcttc ttgagatcgt ttggtctgc gcgtaatctc ttgctctgaa aacgaaaaa	7140
ccgccttgca gggcggtttt tcgaaggttc tctgagctac caactctttg aaccgaggtg	7200
actggccttg aggagcgagc tcacaaaaac ttgtccttcc agtttagcct taaccggcgc	7260
atgacttcaa gactaactcc tctaatacaa ttaccagtgg ctgctgccag tgggtccttt	7320
gcatgtcttt ccgggttgga ctcaagacga tagttaccgg ataaggcgca gcggtcggac	7380
tgaacggggg gttcgtgcat acagtccagc ttggagcgaa ctgcctaccc ggaactgagt	7440
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ggcaggaaca ggagagcgca cgagggagcc gccaggggga aacgcctggt atctttatag	7560
tcctgtcggg ttctgccacc actgatttga gcgtcagatt tcgtgatgct tgtcaggggg	7620
gcggagccta tggaanaacg gctttgccgc ggccctctca ctccctggt aagtatcttc	7680
ctggcatctt ccaggaaatc tccgcccgt tcgtaagcca ttccgctcg ccgcagtcca	7740
acgaccgagc gtatgcgagtc agtgagcgag gaagcggaat atatcctgta tcacatatcc	7800
tgtgacgca ccggtgcagc cttttttctc ctgccacatg aagcacttca ctgacaccct	7860
catcagtccc aacatagtaa gccagtatac actccgctag cgctgatgtc cggcgggtgt	7920
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atcctggtgt cctgttgat accgggaagc cctgggcca cttttggcga aaatgagacg	8160
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ctggatatac caccgttgat atatcccaat ggcacgtaa agaacattt gaggcatttc	8340
agtcagttgc tcaatgtacc tataaccaga ccgttcagct ggatattacg gcctttttaa	8400
agaccgtaaa gaaaaataag cacaagtttt atccggcctt tattcacatt ctgcccgc	8460
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cagccaatcc ctgggtgagt ttaccagtt ttgattttaa cgtggccaat atggacaact	8760
tcttcgcccc cgttttcacc atgggcaaat attatacgca aggcgacaag gtgctgatgc	8820
cgctggcgat tcaggttcat catgccgtct gtgatggctt ccatgtcggc agaagctta	8880
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attggtgccc ttaaacgcct ggtgctacgc ctgaataagt gataataagc ggatgaatgg	9000
cagaaattcg aaagcaaat cgaccgggtc gtcggttcag ggcaggggtc ttaaatagcc	9060
gcttatgtct attgctggtt taccggttta ttgactaccg gaagcagtg gaccgtgtgc	9120
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atgatcattt attctgcctc ccagagcctg ataaaaacgg ttagcgcttc gttaatcacg	9240
atgtaggtgt tccacagggg agccagcagc atcctgcgat gcagatccgg aacataatgg	9300
tgacgggcgc ttgtttcggc gtgggtatgg tggcaggccc cgtggccggg ggactgttgg	9360

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gcgctgccgg cacctgtcct acgagttgca tgataaagaa gacagtcata agtgccggcga 9420
cgatagtcct gccccgcgcc caccggaagg agctaccgga cagcgggtgcg gactgttgta 9480
actcagaata agaaatgagg ccgctcatgg cgttgactct cagtcatagt atcgtggtat 9540
caccgggttg ttccactctc tgttgccggc aacttcagca gcacgtaggg gacttccgcg 9600
tttccagact ttacgaaaca cggaaaccga agaccattca tgttgttgct caggtcgcag 9660
acgttttgca gcagcagtcg cttcacgttc gtcgcgtat cggtgattca ttctgctaac 9720
cagtaaggca accccgccag cctagccggg tcctcaacga caggagcacg atcatgcgca 9780
cccgtggcca ggaccaacg ctgcccg 9808

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<210> SEQ ID NO 34

<211> LENGTH: 1869

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 34

```

atggcattgc caactgcacg tccattacta ggtagttgcg gtacaccagc actaggttct 60
ttattatttt tgtatttttc tctaggttgg gttcaaccaa gtctacatt agcaggtgaa 120
acaggtcaag aagcagcacc acttgacggt gtattaacga atccaccaa tatatcaagt 180
ttaagtccac gtcaattatt aggttttcca tgtgcagaag tticaggttt aagtacagaa 240
cgtgtccggt agttagcagt tgcattagca caaaaaaacg ttaaattatc tacagaacag 300
ttacgttggt tagcccatag attaagcgaa ccaccagaag acttagatgc acttccttta 360
gaccttcttt tattcttaaa tccagatgca ttttcaggac cacaagcatg tacacgtttt 420
tttagtcgaa ttacaaaagc caatgttgat ttattacctc gtggggctcc tgaaagacaa 480
cgtttattac ctgctgcatt agcatgctgg ggtgttcgcg gtagcttatt aagtgaagcc 540
gatgttcgtg ctttaggggg tttagcatgt gatttacctg gtctgttcgt tgcagaatca 600
gcagaagtgt tattaccgag attagtftca tgcccaggac ctttagatca agatcaacaa 660
gaggcagcta gacagctctc tcaaggagga ggcccaccat atggcccacc aagtacatgy 720
agtgtttcta caatggatgc gtaagaggt ttattaccgg ttttaggaca accaattatt 780
cgtagtattc cacaaggcat tgtagcagca tggcgtcaac gtagttctcg tgatccgtct 840
tggcgacaac cagaacgtac aattctacgt ccaagatttc gtagagaagt agaaaaaacg 900
gcgtgtccta tgggcaaaaa agcacgtgaa attgatgaaa gtttaatttt ttataaaaaa 960
tgggaattag aagcatgtgt cgatgcagca ttactagcta cacaaatgga tcgtgttaat 1020
gctattccat tcacatatga acaattagat gttttaaaag ataaattaga cgaattatat 1080
ccacaagggt atccagaatc agttattcaa catttaggtt acttattttt aaaaatgagt 1140
ccagaagaca tacgcaaatg gaatgttaca agtttagaaa cattaaaagc gcttttagaa 1200
gttaacaaag gtcattgaaat gagtccacaa gttgtacgt taattgatag attcgttaaa 1260
ggccgtggtc aattagataa agatacttta gatacattaa cagcatttta tcctggctac 1320
ttatgcagtt tatcaccaga agaattaagt tccgttccac cgagtagtat ctgggcagtt 1380
cgtccgcaag atttagatgc atgcgacca cgtcaattag atgttttata tccaaaagca 1440
agattagctt tccaaaatat gaacggtagt gaatttttcg taaaaattca atccttttta 1500

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gggtggtgcac caactgaaga tctaaaagca ttaagccaac aaaatgtaag tatggattta 1560
gctacgttta tgaaattacg tacagatgca gttctacat taacagttgc agaagttcaa 1620
aaattattag gtccacacgt agaaggatta aaagcagaag aacgtcacg tccagttcgc 1680
gattggattt tacgtcaacg tcaagatgat ttagatacat taggtttagg ttacaaggc 1740
ggtattccga atggtatttt agtgtagat ttatctgttc aagaagcatt aagtgtgata 1800
ccgtgtttat taggtccagg tccagtttta acagtgttag cattattatt agccagtaca 1860
ttagcttaa 1869

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<210> SEQ ID NO 35
<211> LENGTH: 622
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 35

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Met Ala Leu Pro Thr Ala Arg Pro Leu Leu Gly Ser Cys Gly Thr Pro
 1             5             10             15
Ala Leu Gly Ser Leu Leu Phe Leu Leu Phe Ser Leu Gly Trp Val Gln
 20             25             30
Pro Ser Arg Thr Leu Ala Gly Glu Thr Gly Gln Glu Ala Ala Pro Leu
 35             40             45
Asp Gly Val Leu Thr Asn Pro Pro Asn Ile Ser Ser Leu Ser Pro Arg
 50             55             60
Gln Leu Leu Gly Phe Pro Cys Ala Glu Val Ser Gly Leu Ser Thr Glu
 65             70             75             80
Arg Val Arg Glu Leu Ala Val Ala Leu Ala Gln Lys Asn Val Lys Leu
 85             90             95
Ser Thr Glu Gln Leu Arg Cys Leu Ala His Arg Leu Ser Glu Pro Pro
100            105            110
Glu Asp Leu Asp Ala Leu Pro Leu Asp Leu Leu Leu Phe Leu Asn Pro
115            120            125
Asp Ala Phe Ser Gly Pro Gln Ala Cys Thr Arg Phe Phe Ser Arg Ile
130            135            140
Thr Lys Ala Asn Val Asp Leu Leu Pro Arg Gly Ala Pro Glu Arg Gln
145            150            155            160
Arg Leu Leu Pro Ala Ala Leu Ala Cys Trp Gly Val Arg Gly Ser Leu
165            170            175
Leu Ser Glu Ala Asp Val Arg Ala Leu Gly Gly Leu Ala Cys Asp Leu
180            185            190
Pro Gly Arg Phe Val Ala Glu Ser Ala Glu Val Leu Leu Pro Arg Leu
195            200            205
Val Ser Cys Pro Gly Pro Leu Asp Gln Asp Gln Gln Glu Ala Ala Arg
210            215            220
Ala Ala Leu Gln Gly Gly Gly Pro Pro Tyr Gly Pro Pro Ser Thr Trp
225            230            235            240
Ser Val Ser Thr Met Asp Ala Leu Arg Gly Leu Leu Pro Val Leu Gly
245            250            255
Gln Pro Ile Ile Arg Ser Ile Pro Gln Gly Ile Val Ala Ala Trp Arg
260            265            270
Gln Arg Ser Ser Arg Asp Pro Ser Trp Arg Gln Pro Glu Arg Thr Ile
275            280            285

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Leu	Arg	Pro	Arg	Phe	Arg	Arg	Glu	Val	Glu	Lys	Thr	Ala	Cys	Pro	Ser
290						295					300				
Gly	Lys	Lys	Ala	Arg	Glu	Ile	Asp	Glu	Ser	Leu	Ile	Phe	Tyr	Lys	Lys
305					310					315				320	
Trp	Glu	Leu	Glu	Ala	Cys	Val	Asp	Ala	Ala	Leu	Leu	Ala	Thr	Gln	Met
				325						330				335	
Asp	Arg	Val	Asn	Ala	Ile	Pro	Phe	Thr	Tyr	Glu	Gln	Leu	Asp	Val	Leu
			340					345					350		
Lys	His	Lys	Leu	Asp	Glu	Leu	Tyr	Pro	Gln	Gly	Tyr	Pro	Glu	Ser	Val
		355					360					365			
Ile	Gln	His	Leu	Gly	Tyr	Leu	Phe	Leu	Lys	Met	Ser	Pro	Glu	Asp	Ile
370						375					380				
Arg	Lys	Trp	Asn	Val	Thr	Ser	Leu	Glu	Thr	Leu	Lys	Ala	Leu	Leu	Glu
385				390						395					400
Val	Asn	Lys	Gly	His	Glu	Met	Ser	Pro	Gln	Val	Ala	Thr	Leu	Ile	Asp
			405						410					415	
Arg	Phe	Val	Lys	Gly	Arg	Gly	Gln	Leu	Asp	Lys	Asp	Thr	Leu	Asp	Thr
			420					425					430		
Leu	Thr	Ala	Phe	Tyr	Pro	Gly	Tyr	Leu	Cys	Ser	Leu	Ser	Pro	Glu	Glu
		435					440					445			
Leu	Ser	Ser	Val	Pro	Pro	Ser	Ser	Ile	Trp	Ala	Val	Arg	Pro	Gln	Asp
	450					455					460				
Leu	Asp	Thr	Cys	Asp	Pro	Arg	Gln	Leu	Asp	Val	Leu	Tyr	Pro	Lys	Ala
465					470					475				480	
Arg	Leu	Ala	Phe	Gln	Asn	Met	Asn	Gly	Ser	Glu	Tyr	Phe	Val	Lys	Ile
			485					490						495	
Gln	Ser	Phe	Leu	Gly	Gly	Ala	Pro	Thr	Glu	Asp	Leu	Lys	Ala	Leu	Ser
			500					505					510		
Gln	Gln	Asn	Val	Ser	Met	Asp	Leu	Ala	Thr	Phe	Met	Lys	Leu	Arg	Thr
		515					520					525			
Asp	Ala	Val	Leu	Pro	Leu	Thr	Val	Ala	Glu	Val	Gln	Lys	Leu	Leu	Gly
	530					535					540				
Pro	His	Val	Glu	Gly	Leu	Lys	Ala	Glu	Glu	Arg	His	Arg	Pro	Val	Arg
545					550					555				560	
Asp	Trp	Ile	Leu	Arg	Gln	Arg	Gln	Asp	Asp	Leu	Asp	Thr	Leu	Gly	Leu
			565					570						575	
Gly	Leu	Gln	Gly	Gly	Ile	Pro	Asn	Gly	Tyr	Leu	Val	Leu	Asp	Leu	Ser
			580					585					590		
Val	Gln	Glu	Ala	Leu	Ser	Gly	Thr	Pro	Cys	Leu	Leu	Gly	Pro	Gly	Pro
		595					600					605			
Val	Leu	Thr	Val	Leu	Ala	Leu	Leu	Leu	Ala	Ser	Thr	Leu	Ala		
	610					615					620				

<210> SEQ ID NO 36

<211> LENGTH: 1878

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 36

atggcattac caacggtctg cccattatta gggtcttctg gttcaccaat ttgtatctgc 60

agttttttat tattattact atcttttaggt tggattccgc gtttacaac acaaaccact 120

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aaaacaagtc aagaagctac attattgcat gcagtcgaatg gcgcagcaga ttttgcaagt 180
ttaccaacag gcttatttct tggctcttaca tgtgaagaag ttagtgattt aagtatggaa 240
caagcaaaag gtttagcgat ggcggttcgc caaaaaata ttacattacg tggcatcaa 300
ttacgttggt tagcacgctg tttaccacga catttaacag atgaagaatt aaatgctcta 360
ccattagact tattattatt tttaaatcca gcaatgtttc caggtaaca agcatgtgcc 420
cattttttca gtttaatttc gaaagcaaet gtagatgttt taccgagacg tagcttagaa 480
cgtcaacgtc ttttaattga agcattaaaa tgtcaagggt tttatgggtt ccaagttagt 540
gaagcagatg ttcgtgcact tgggtgttta gcttggtatt taccagggaa attttagtga 600
cgttctagtg aagtattatt accatgggta gcagggtgtc aagggtccatt agatcaaagt 660
caagaaaaag cagttcgtga agtcttacgt agtggtcgta ctcaatatgg cccacctagc 720
aatggagtg ttagtaccgt agatgcatta caaagtttag tagctgtttt agatgaaagt 780
attgttcaga gtattccaaa agatgtgaaa gcagagtggg tacaacatat ttcccgtgac 840
ccatctcggt taggtagtaa attaacagtt attcatccac gttttcgccg cgacgcagaa 900
caaaaagcat gtccaccagg taaagaacca tataaagtag atgaagattt aattttttat 960
cagaattggg aattagaagc ctgtgttgat ggtacaatgt tagcacgtca aatggattta 1020
gtaaatgaaa ttccatttac atatgaacaa ttaagtatct ttaaacataa attagataaa 1080
acatatccac aaggttatcc agaatcggtt attcaacaat taggtcattt ttttcgttat 1140
gtagtccag aagacattca tcaatggaat gttacaagtc cagatcacgt taaaacttta 1200
ttaaagttta gtaaaggcca aaaaatgaat gctcaagcaa ttgcattagt cgcattgtat 1260
ttacgtggag gtggccaatt agatgaagat atggttaaag cattagggga tattccatta 1320
tcatatttat gtgattttct cccacaagac ttacattcag ttccaagtag tgttatgtgg 1380
ttagttggtc cacaagggtt agataaatgt agtcaacgtc atttaggtt actttatcaa 1440
aaagcatgta gtgcgtttca aaatgttagt ggtttagaat attttgaaaa aatcaaaaca 1500
tttttaggag gtgcatctgt aaaagattta gcgcattaa gtcaacataa tgtaagtatg 1560
gatatcgcaa catttaaacy tttacaagtc gatagtctag ttggtcttag tgtacgagaa 1620
gttcaaaat tattagggcc gaatattgta gatttaaaaa cagaagaaga taaaagtcca 1680
gttcgtgact ggttatttcg acaacatcag aaagacttag atcgtcttgg attaggttta 1740
caagggtgta ttccaaatgg ttatttagtt ttagatttta atgtacgtga agcatttagt 1800
tcaagagcga gtttattagg tccagggttt gtgttaattt ggattccagc attactacca 1860
gcacttcggt tatcataa 1878

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<210> SEQ ID NO 37
 <211> LENGTH: 625
 <212> TYPE: PRT
 <213> ORGANISM: Mus musculus

<400> SEQUENCE: 37

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Met Ala Leu Pro Thr Ala Arg Pro Leu Leu Gly Ser Cys Gly Ser Pro
 1             5             10             15
Ile Cys Ser Arg Ser Phe Leu Leu Leu Leu Ser Leu Gly Trp Ile
      20             25             30
Pro Arg Leu Gln Thr Gln Thr Thr Lys Thr Ser Gln Glu Ala Thr Leu

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35	40	45
Leu His Ala Val Asn Gly Ala Ala Asp Phe Ala Ser Leu Pro Thr Gly		
50	55	60
Leu Phe Leu Gly Leu Thr Cys Glu Glu Val Ser Asp Leu Ser Met Glu		
65	70	75
Gln Ala Lys Gly Leu Ala Met Ala Val Arg Gln Lys Asn Ile Thr Leu		
85	90	95
Arg Gly His Gln Leu Arg Cys Leu Ala Arg Arg Leu Pro Arg His Leu		
100	105	110
Thr Asp Glu Glu Leu Asn Ala Leu Pro Leu Asp Leu Leu Leu Phe Leu		
115	120	125
Asn Pro Ala Met Phe Pro Gly Gln Gln Ala Cys Ala His Phe Phe Ser		
130	135	140
Leu Ile Ser Lys Ala Asn Val Asp Val Leu Pro Arg Arg Ser Leu Glu		
145	150	155
Arg Gln Arg Leu Leu Met Glu Ala Leu Lys Cys Gln Gly Val Tyr Gly		
165	170	175
Phe Gln Val Ser Glu Ala Asp Val Arg Ala Leu Gly Gly Leu Ala Cys		
180	185	190
Asp Leu Pro Gly Lys Phe Val Ala Arg Ser Ser Glu Val Leu Leu Pro		
195	200	205
Trp Leu Ala Gly Cys Gln Gly Pro Leu Asp Gln Ser Gln Glu Lys Ala		
210	215	220
Val Arg Glu Val Leu Arg Ser Gly Arg Thr Gln Tyr Gly Pro Pro Ser		
225	230	235
Lys Trp Ser Val Ser Thr Leu Asp Ala Leu Gln Ser Leu Val Ala Val		
245	250	255
Leu Asp Glu Ser Ile Val Gln Ser Ile Pro Lys Asp Val Lys Ala Glu		
260	265	270
Trp Leu Gln His Ile Ser Arg Asp Pro Ser Arg Leu Gly Ser Lys Leu		
275	280	285
Thr Val Ile His Pro Arg Phe Arg Arg Asp Ala Glu Gln Lys Ala Cys		
290	295	300
Pro Pro Gly Lys Glu Pro Tyr Lys Val Asp Glu Asp Leu Ile Phe Tyr		
305	310	315
Gln Asn Trp Glu Leu Glu Ala Cys Val Asp Gly Thr Met Leu Ala Arg		
325	330	335
Gln Met Asp Leu Val Asn Glu Ile Pro Phe Thr Tyr Glu Gln Leu Ser		
340	345	350
Ile Phe Lys His Lys Leu Asp Lys Thr Tyr Pro Gln Gly Tyr Pro Glu		
355	360	365
Ser Leu Ile Gln Gln Leu Gly His Phe Phe Arg Tyr Val Ser Pro Glu		
370	375	380
Asp Ile His Gln Trp Asn Val Thr Ser Pro Asp Thr Val Lys Thr Leu		
385	390	395
Leu Lys Val Ser Lys Gly Gln Lys Met Asn Ala Gln Ala Ile Ala Leu		
405	410	415
Val Ala Cys Tyr Leu Arg Gly Gly Gly Gln Leu Asp Glu Asp Met Val		
420	425	430
Lys Ala Leu Gly Asp Ile Pro Leu Ser Tyr Leu Cys Asp Phe Ser Pro		
435	440	445

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Gln Asp Leu His Ser Val Pro Ser Ser Val Met Trp Leu Val Gly Pro
 450 455 460
 Gln Gly Leu Asp Lys Cys Ser Gln Arg His Leu Gly Leu Leu Tyr Gln
 465 470 475 480
 Lys Ala Cys Ser Ala Phe Gln Asn Val Ser Gly Leu Glu Tyr Phe Glu
 485 490 495
 Lys Ile Lys Thr Phe Leu Gly Gly Ala Ser Val Lys Asp Leu Arg Ala
 500 505 510
 Leu Ser Gln His Asn Val Ser Met Asp Ile Ala Thr Phe Lys Arg Leu
 515 520 525
 Gln Val Asp Ser Leu Val Gly Leu Ser Val Ala Glu Val Gln Lys Leu
 530 535 540
 Leu Gly Pro Asn Ile Val Asp Leu Lys Thr Glu Glu Asp Lys Ser Pro
 545 550 555 560
 Val Arg Asp Trp Leu Phe Arg Gln His Gln Lys Asp Leu Asp Arg Leu
 565 570 575
 Gly Leu Gly Leu Gln Gly Gly Ile Pro Asn Gly Tyr Leu Val Leu Asp
 580 585 590
 Phe Asn Val Arg Glu Ala Phe Ser Ser Arg Ala Ser Leu Leu Gly Pro
 595 600 605
 Gly Phe Val Leu Ile Trp Ile Pro Ala Leu Leu Pro Ala Leu Arg Leu
 610 615 620
 Ser
 625

<210> SEQ ID NO 38
 <211> LENGTH: 335
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Partial expression cassette comprising codon-
 optimized sequence

<400> SEQUENCE: 38

gtacctcctt tgattagtat attcctatct taaagttact tttatgtgga ggcattaaca 60
 tttgttaatg acgtcaaaag gatagcaaga ctagaataaa gctataaagc aagcatataa 120
 tattgcgttt catctttaga agcgaatttc gccaatatta taattatcaa aagagagggg 180
 tggcaaacgg tatttggcat tattaggtta aaaaatgtag aaggagagtg aaacctatga 240
 aaaaacgtaa agttttaatt ccattaatgg cattaagtac aattttagtt agtagtacag 300
 gtaatttaga agttattcaa gcagaagttg gatcc 335

<210> SEQ ID NO 39
 <211> LENGTH: 2778
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Coding sequences of plasmid comprising codon-
 optimized sequence

<400> SEQUENCE: 39

ggaatccaag gaaaagaagt cgtactttta gatttcgcag cagcaggagg agaattagga 60
 tggttaactc atccatatgg caaaggctgg gatttaatgc aaaacattat gaacgatatg 120
 ccaatttata tgtactccgt atgtaatgta atgagcgggtg atcaagataa ctggttacgt 180

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actaattggg tttatcgagg tgaagcagaa agaattttta ttgaacttaa atttactgtt	240
cgtgactgta atagttttcc aggaggggcc tcatcatgta aagaacatt caatctatat	300
tatgccgaaa gcgatcttga ttatggtaca aatttccaaa aacgtttatt tactaaaatt	360
gatacaatag ctccagatga aatcactgta agttccgatt ttgaagctcg tcatgtaaaa	420
ttaaatgtag aagaacgcag tgttggtcca ctaactagaa aaggatttta tcttgctttc	480
caagatatag gggcttgcgt agcattgtta tccgttcgtg tatactataa aaaatgtcca	540
gaactacttc aaggcttagc acattttcca gaaacaattg cgggctcaga tgcgccatca	600
cttgcaactg tggcgggtac atgtgttgat catgctgtt tgcaccagg aggagaggaa	660
cctcgcatgc actgtgcagt agatggtgaa tggttagttc ctattggta atgtttatgt	720
caagccggtt atgaaaaagt tgaagatgct tgtcaagcat gctcccagg tttttttaa	780
ttcgaagcta gtgaatctcc atgcttagaa tgtccagaac acacattacc aagtcagaa	840
ggtgcaactg cctgtgaatg cgaagaaggt ttttttcgtg cccacaaga tccagcctca	900
atgccttgta cagcaccgcc ttctgctcca cactatttaa cagccgtagg aatgggcgct	960
aaagtagagt tacgatggac accgcctcaa gatagtggag gcogtgaaga tattgtttat	1020
tccgttactt gtgaacaatg ctggccagaa agtggtgaat gcgggccttg cgaagcatca	1080
gtagatatt cggaaccacc acacgggtta actagaacta gtgtcacagt atcagactta	1140
gaaccacaca tgaattatac atttacagtt gaggcacgta atggagtatc tggtttagtt	1200
acatcacgct cttttcgac agcatcggtc tctattaacc aaactgaacc gccaaaagta	1260
agattagaag ggcgttcgac aacatcactt tccgtaagtt ggtcaattcc accaccacaa	1320
caatcacgag tttggaaaa tgaagttaca tacagaaaaa aaggagattc gaatagtatt	1380
aatgttagac gtacagaagg attcagcgta accctagatg atttagctcc agatacaaca	1440
tatttagtac aggtgcaagc attaacacaa gaaggacaag gggcgggctc acgagttcat	1500
gaatttcaaa cattacatag aagaagaaa aatcaaagag cagctcaatc accagaagat	1560
gtttattttt caaagtctga acagttgaaa ccattgaaaa cctatgttga tccacacaca	1620
tacgaagacc caaaccaagc ggtccttaaa tttaaacccg aaattcatcc atcatcgta	1680
actcgtaaaa aagtgatcgg agctggagaa ttcggggagg tatacaagg catgttgaaa	1740
acctcaagtg gtaaaaaaga agttcctgta gcaattatga ctcttaaagc agggatatac	1800
gaaaaacaac gagttgattt tttaggcgaa gctggtatca tgggacaatt ttcgcatcat	1860
aataaatta gacttgaagg tgttatctct aaatataaac caatgatgat tattactgaa	1920
tatatgaaa acggtgcttt agataaattt ctacgcgaaa aagatggtga attttctgtc	1980
cttcaattag ttggtatgtt acgtggcatc gctgcaggta tgaatatatc tgccaacatg	2040
aattatgtac atagagattt agcggctcga aatattcttg taaattccaa tttagtgtgc	2100
aaagttagtg atttcggttt aagtcagta ttagaagatg atccagaagc aacctatact	2160
acttcggggg gtaaaattcc gatccgttg acagcaccgg aagcaatttc atatcgtaaa	2220
tttacatctg caagcgatgt ttggagtctt ggaattgtga tgtgggaagt aatgacatac	2280
ggcgaacgtc catattggga attgtcaaac catgaagtaa tgaagcgat taacgatggt	2340
ttcagattac caacccaat ggactgtcca tcagcaattt atcaactaat gatgcaatgc	2400
tggcaacaag aaagagctag aagacctaaa tttgcagaca ttgtttcaat tttagacaaa	2460

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ctaattcgtg cgccagatag tcttaaaacc ctactgtgatt tcgatccacg cgtatcaatt	2520
cgtcttccat caacatcggg atctgaagggt gttcctttta gaacagtaag cgagtgggta	2580
gaatcgatta aaatgcaaca gtatacagaa cattttatgg cagccggata cacagcaatt	2640
gaaaaagttg tgcaaatgac aaatgatgat attaaacgta ttggagtgcg tctacctggc	2700
cacaaaaaac gtattgtcta ctccctttta ggtttaaag accaagtaaa tacagtcgga	2760
attccaatat gagagctc	2778

<210> SEQ ID NO 40
 <211> LENGTH: 1382
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Subfragment of codon-optimized sequence
 containing intergenic region

<400> SEQUENCE: 40

acgcgttttg aaatatgaag ttacatacag aaaaaaagga gattcgaata gttataatgt	60
tagacgtaca gaaggattca gcgtaaccct agatgattta gctccagata caacatattt	120
agtacagggt caagcattaa cacaagaagg acaaggggag ggctcacgag ttcataat	180
tcaaacatta taaaaacaca gaacgaaga aaaagtgaag tgaatgatat ggcatatgat	240
agtcgttttg atgaatgggt tcaaaaatta aaagaagaaa gttttcaaaa taatacattt	300
gatcgtcgtg aattttatca aggtgcagggt aaaattgcag gtttaagttt aggtttaaca	360
attgcacaaa gtgttggtgc atttcataga agaagaaga atcaaaagag acgtcaatca	420
ccagaagatg tttatttttc aaagtctgaa cagttgaaac cattgaaac ctatgttgat	480
ccacacacat acgaagaccc aaaccaagcg gtccttaaat ttacaaccga aattcatcca	540
tcatgcgtaa ctgcgtcaaaa agtgatcgga gctggagaat tcggggagggt atacaaggc	600
atgttgaaaa cctcaagtgg taaaaaagaa gttcctgtag caattatgac tcttaaagca	660
gggtatacag aaaaaacaag agttgatatt ttaggcgaag ctggtatcat gggacaattt	720
tcgcatcata atataattag acttgaagggt gttatctcta aatataaacc aatgatgatt	780
attactgaat atatggaaaa cggtgcttta gataaatttc tacgcgaaaa agatggtgaa	840
ttttctgtcc ttcaattagt tggatatgta cgtggcatcg ctgcagggtat gaaatatctt	900
gccaacatga attatgtaca tagagattta gcggctcgaa atattcttgt aaattccaat	960
ttagtgtgca aagtttagta tttcggttta agtcgagtat tagaagatga tccagaagca	1020
acctatacta ctccggggg taaaattccg atccgttgga cagcaccgga agcaatttca	1080
tatcgtaaat ttacatctgc aagcgatgtt tggagtctcg gaattgtgat gtgggaagta	1140
atgacatacg gcgaacgtcc atattgggaa ttgtcaaac atgaagtaat gaaagcgatt	1200
aacgatggtt tcagattacc aaccccaatg gactgtccat cagcaattta tcaactaatg	1260
atgcaatgct ggcaacaaga aagagctaga agacctaat ttgcagacat tgtttcaatt	1320
ttagacaaac taattcgtgc gccagatagt cttaaaaccc tagctgattt cgatccacgc	1380
gt	1382

<210> SEQ ID NO 41
 <211> LENGTH: 447
 <212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic construct

<400> SEQUENCE: 41

```
ggtacctcct ttgattagta tattcctatc ttaaagttac ttttatgtgg aggcattaac    60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata    120
atattgcgtt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtatttggca ttattagggt aaaaaatgta gaaggagagt gaaacccatg    240
aatatgaaaa aagctacgat tgcagctaca gccggcattg ccgtaacagc ttttgagca    300
ccaactattg cctcagcctc tacagttggt gtcgaagcag gagacacatt atggggaatc    360
gcacaatcaa aaggtacaac ggttgatgct attaaaaaag cgaataattt aacaacagat    420
aaaatcgtgc caggtaaaaa actgcag                                     447
```

<210> SEQ ID NO 42

<211> LENGTH: 1683

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Plasmid subfragment

<400> SEQUENCE: 42

```
ggtacctcct ttgattagta tattcctatc ttaaagttac ttttatgtgg aggcattaac    60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata    120
atattgcgtt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg    180
gtggcaaacg gtatttggca ttattagggt aaaaaatgta gaaggagagt gaaacccatg    240
aatatgaaaa aagctacgat tgcagctaca gccggcattg ccgtaacagc ttttgagca    300
ccaactattg cctcagcctc tacagttggt gtcgaagcag gagacacatt atggggaatc    360
gcacaatcaa aaggtacaac ggttgatgct attaaaaaag cgaataattt aacaacagat    420
aaaatcgtgc caggtaaaaa actgcaggta aataatgagg ttgctgctgc tgaaaaaaca    480
gagaaatctg ttacgcgaac ttggttaaac gtccgtactg gcgctggtgt tgataacagt    540
attattacgt ccatcaaaag tggaacaaaa gtaactgttg aaacaaccga atctaacggc    600
tggcacaaaa ttacttacaa cgatggaaaa actgggtttcg ttaacggtaa atacttaact    660
gacaaagcag taagcactcc agttgcacca acacaagaag tgaaaaaaga aactactact    720
caacaagctg cacctgttgc agaaacaaaa actgaagtaa aacaaactac acaagcaact    780
acacctgcgc ctaaagtagc agaaacgaaa gaaactccag taatagatca aaatgctact    840
acacacgctg tcaaaagcgg tgacactatt tgggctttat ccgtaaaata cgggtgtttc    900
gttcaagaca ttatgtcatg gaataattta tcttcttctt ctatttatgt aggtcaaaag    960
cttgctatta aacaaactgc taacacagct actccaaaag cagaagtga aacgggaagct   1020
ccagcagctg aaaaacaagc agctccagta gttaaagaaa atactaacac aaatactgct   1080
actacagaga aaaaagaaac agcaacgcaa caacaaacag cacctaaagc accaacaagaa   1140
gtgcacaaac cagctcctgc accatctaca aacacaaatg ctaataaaac gaatacaaat   1200
acaaatacaa acaatactaa tacaccatct aaaaaacta atacaaactc aaatactaat   1260
acgaatacaa actcaaatc gaatgcta atcgaatcct ccaacaataa cagcaattca   1320
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agtgcagtg ctattattgc tgaagctcaa aaacaccttg gaaaagctta ttcattgggt 1380
ggtaacggac caactacatt tgattgctct ggttacctta aatatgtatt tgctaaagcg 1440
ggtatctccc ttccacgtac atctggcgca caatatgcta gcactacaag aatttctgaa 1500
tctcaagcaa aacctgggtga tttagtattc ttcgactatg gtacgggaat ttctcacatt 1560
ggtatttatg ttggtaatgg tcaaatgatt aacgcgcaag acaatggcgt taaatacgat 1620
aacatccacg gctctggctg gggtaaatat ctagtgggct tcggtcgcgt ataataagga 1680
tcc 1683

```

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<210> SEQ ID NO 43
<211> LENGTH: 3552
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Plasmid subfragment

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<400> SEQUENCE: 43

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ggtaacctct ttgattagta tattcctatc ttaaagttac ttttatgtgg aggcattaac 60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata 120
atattgcgtt tcattctttag aagcgaattt cgccaatatt ataattatca aaagagaggg 180
gtggcaaacg gtatttggtc ttattaggtt aaaaaatgta gaaggagagt gaaacccatg 240
aatatgaaaa aaggtacgat tgcagctaca gccggcattg ccgtaacagc ttttgcagca 300
ccaactattg cctcagcctc tacagttggt gtcgaagcag gagacacatt atggggaatc 360
gcacaatcaa aaggtacaac ggttgatgct attaaaaaag cgaataatct aacaacagat 420
aaaaatcggtc caggtcaaaa actgcaggca ttgccactg cactgccatt actaggtagt 480
tgcggtacac cagcactagg ttctttatta tttttgttat tttctctagg ttgggttcaa 540
ccaagtctga cattagcagg tgaacacagt caagaagcag caccacttga cgggtgtatta 600
acgaatccac caaatatatac aagtttaagt ccacgtcaat tattaggttt tccatgtgca 660
gaagtttcag gtttaagtac agaacgtgtc cgtgagttag cagttgcatt agcacaacaaa 720
aacgttaaat tatctacaga acagttacgt tgtttagccc atagattaag cgaaccacca 780
gaagacttag atgcacttcc ttttagacct cttttattct taaatccaga tgcattttca 840
ggaccacaag catgtacacg tttttttagt cgaattacaa aagccaatgt tgatttatta 900
cctcgtgggg ctctgaaag acaacgttta ttacctgctg cattagcatg ctggggtggt 960
cgcggtagct tattaagtga agccgatgtt cgtgctttag ggggtttagc atgtgattta 1020
cctggtcggt tcgttcgaga atcagcagaa gtgttattac cgagattagt ttcattgcca 1080
ggacctttag atcaagatca acaagaggca gctagagcag ctcttcaagg aggaggccca 1140
ccatatggcc caccaagtac atggagtgtt tctacaatgg atgcgttaag aggtttatta 1200
ccggttttag gacaaccaat tattcgtagt attccacaag gcattgtagc agcatggcgt 1260
caacgtagtt ctctgtatcc gtcttggcga caaccagaac gtacaattct acgtccaaga 1320
tttcgtagag aagtagaaaa aacggcgtgt cctagtggca aaaaagcacg tgaaattgat 1380
gaaagttaa ttttttataa aaaatgggaa ttagaagcat gtgtcgatgc agcattacta 1440
gctacacaaa tgagtcgtgt taatgctatt ccattcacat atgaacaatt agatgtttta 1500
aagcataaat tagacgaatt atatccacaa gggtatccag aatcagttat tcaacattta 1560

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ggttacttat ttttaaaat gagtccagaa gacatacgca aatggaatgt tacaagttta	1620
gaaacattaa aagcgctttt agaagttaac aaaggatcatg aatgagatcc acaagttgct	1680
acgttaattg atagattcgt taaaggccgt ggtcaattag ataaagatac tttagataca	1740
ttacagcat tttatcctgt ctacttatgc agtttatcac cagaagaatt aagttccgtt	1800
ccaccgagta gtatctgggc agttcgtccg caagatttag atacatgcga cccacgtcaa	1860
ttagatgttt tatatccaaa agcaagatta gctttccaaa atatgaacgg tagtgaatat	1920
ttcgtaaaaa ttcaatcctt tttagtggtt gcaccaactg aagatctaaa agcattaagc	1980
caacaaaatg taagtatgga tttagctacg tttatgaaat tacgtacaga tgcagttcta	2040
ccattaacag ttgcagaagt tcaaaaatta ttaggtccac acgtagaagg attaaaagca	2100
gaagaacgtc accgtccagt tcgcgattgg attttacgtc aacgtcaaga tgatttagat	2160
acattaggtt taggtttaca aggcggtatt ccgaatggat atttagtggt agatttatct	2220
gttcaagaag cattaagtgg tacaccgtgt ttattaggtc caggtccagt ttaacagtg	2280
ttagcattat tattagccag tacattagct ctgcaggtaa ataagaggt tgctgctgct	2340
gaaaaaacag agaaatctgt tagcgcaact tggttaaacg tccgtactgg cgctggtgtt	2400
gataacagta ttattacgct catcaaaggt ggaacaaaag taactgttga aacaaccgaa	2460
tctaacggct ggcacaaaaa tacttacaac gatggaaaaa ctggtttcgt taacggtaaa	2520
tacttaactg acaaaagcgt aagcactcca gttgcaccaa cacaagaagt gaaaaaagaa	2580
actactactc aacaagctgc acctgttgca gaaacaaaaa ctgaagtaaa acaactaca	2640
caagcaacta cacctcgccc taaagtagca gaaacgaaag aaactccagt aatagatcaa	2700
aatgctacta cacacgctgt caaaagcggg gacactatct gggctttatc cgtaaaaatc	2760
gggtgtttctg ttcaagacat tatgtcatgg aataatttat cttcttcttc tatttatgta	2820
gggtcaaaagc ttgctattaa acaaactgct aacacagcta ctccaaaagc agaagtgaag	2880
acggaagctc cagcagctga aaaacaagca gctccagtag ttaaagaaaa tactaacaca	2940
aatactgcta ctacagagaa aaagaaga gcaacgcaac aacaacagc acctaagca	3000
ccaacagaag ctgcaaaacc agctcctgca ccactctaca acacaaatgc taataaaacg	3060
aatacaata caaatacaaa caatactaata acaccatcta aaaaactaa tacaactca	3120
aatactaata cgaatacaaa ctcaaatagc aatgctaata aaggttcttc caacaataac	3180
agcaattcaa gtgcaagtgc tattattgct gaagctcaaa aacacctggg aaaagcttat	3240
tcattgggtg gtaacggacc aactacattt gattgctctg gttacactaa atatgtattt	3300
gctaaaagcg gtatctccct tccacgtaca tctggcgcac aatatgctag cactacaaga	3360
attttctgaat ctcaagcaaa acctggtgat ttagtattct tcgactatgg tagcggaaat	3420
tctcacattg gtatttatgt tggtaatggt caaatgatta acgcgcaaga caatggcggt	3480
aaatacgata acatccacgg ctctggctgg ggtaaatatc tagttggctt cggctcgcta	3540
taataaggat cc	3552

<210> SEQ ID NO 44
 <211> LENGTH: 3327
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Plasmid subfragment

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<400> SEQUENCE: 44

ggtacctcct ttgattagta tattcctatc ttaaagtac ttttatgtgg aggcattaac	60
atttgtaaat gacgtcaaaa ggatagcaag actagaataa agctataaag caagcatata	120
atattgcgtt tcatctttag aagcgaattt cgccaatatt ataattatca aaagagaggg	180
gtggcaaacg gtatttgga ttattaggtt aaaaaatga gaaggagagt gaaacccatg	240
aatatgaaaa aagctacgat tgcagctaca gccggcattg ccgtaacagc ttttgagca	300
ccaactattg cctcagcctc tacagttgtt gtcgaagcag gagacacatt atggggaatc	360
gcacaatcaa aaggtacaac ggttgatgct attaaaaag cgaataattt aacaacagat	420
aaaatcgtgc caggtcaaaa actgcagcgt acattagcag gtgaaacagg tcaagaagca	480
gcaccacttg acgggtgatt aacgaatcca ccaatatat caagttaag tccacgtcaa	540
ttattaggtt ttccattgac agaagtttca ggtttaagta cagaacgtgt ccgtgagtta	600
gcagttgcat tagcacaaaa aaacgttaaa ttatctacag aacagttacg ttgtttagcc	660
catagattaa gcgaaccacc agaagactta gatgcacttc ctttagacct tcttttattc	720
ttaaatccag atgcattttc aggaccacaa gcattgtcac gtttttttag tcgaattaca	780
aaagccaatg ttgatttatt acctcgtggg gctcctgaaa gacaacgttt attacctgct	840
gcattagcat gctggggtgt tcgcggtagc ttattaagtg aagccgatgt tcgtgcttta	900
gggggttttag catgtgattt acctggtcgt ttcgttgtag aatcagcaga agtgttatta	960
ccgagattag tttcatgcc ccaggacctta gatcaagatc aacaagaggc agctagagca	1020
gctcttcaag gaggaggccc accatatggc ccaccaagta catggaggtt ttctacaatg	1080
gatgcgttaa gaggtttatt accgggttta ggacaaccaa ttattcgtag tattccacaa	1140
ggcattgtag cagcatggcg tcaacgtagt tctcgtgac cgtcttgccg acaaccagaa	1200
cgtacaattc tacgtccaag atttcgtaga gaagtagaaa aaacggcgtg tcctagtggc	1260
aaaaaagcac gtgaaattga tgaaagtta attttttata aaaaatggga attagaagca	1320
tgtgtcagtg cagcattact agctacacaa atggatcgtg ttaatgctat tccattcaca	1380
tatgaacaat tagatgtttt aaagcataaa tttagcgaat tatatccaca aggttatcca	1440
gaatcagtta ttcaacattt aggttactta tttttaaaa tgagtccaga agacatacgc	1500
aatggaatg ttacaagttt agaaacatta aaagcgcttt tagaagttaa caaaggtcat	1560
gnaatgagtc cacaagtgc tacgttaatt gatagattcg ttaaaggccg tggtaatta	1620
gataaagata ctttagatag attaacagca ttttatcctg gctacttatg cagtttatca	1680
ccagaagaat taagttccgt tccaccgagt agtatctggg cagttcgtcc gcaagattta	1740
gatacatgcg acccagctca attagatgtt ttatatccaa aagcaagatt agctttccaa	1800
aatatgaacg gtagtgaata tttcgtaaaa attcaatcct ttttaggtgg tgcaccaact	1860
gaagatctaa aagcattaag ccaacaaaat gtaagtatgg atttagctac gtttatgaaa	1920
ttacgtacag atgcagttct accattaaca gttgcagaag ttcaaaaatt attaggtcca	1980
cacgtagaag gattaaaagc agaagaacgt caccgtccag ttcgcgattg gattttacgt	2040
caacgtcaag atgatttaga tacattaggt ttaggtttac aaggcctgca ggtaaataat	2100
gaggttgctg ctgctgaaaa aacagagaaa tctgttagcg caacttggtt aaacgtccgt	2160
actggcgctg gtgttgataa cagtattatt acgtccatca aaggtggaac aaaagtaact	2220

-continued

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gttgaacaa ccgaatctaa cggctggcac aaaattactt acaacgatgg aaaaactggt 2280
ttcgttaacg gtaaatactt aactgacaaa gcagtaagca ctccagttgc accaacacaa 2340
gaagtgaaaa aagaaactac tactcaacaa gctgcacctg ttgcagaaac aaaaactgaa 2400
gtaaaacaaa ctacacaagc aactacacct gcgcctaaag tagcagaaac gaaagaaact 2460
ccagtaatag atcaaatgc tactacacac gctgtcaaaa gcggtgacac tatttgggct 2520
ttatccgtaa aatacgggtg ttctgttcaa gacattatgt catggaataa tttatcttct 2580
tcttctatct atgtagggtc aaagcttgct attaaacaaa ctgctaacac agctactcca 2640
aaagcagaag tgaacacgga agtccagca gctgaaaaac aagcagctcc agtagttaa 2700
gaaaatacta acacaaatc tgctactaca gagaaaaaag aaacagcaac gcaacaacaa 2760
acagcaccta aagcaccaac agaagctgca aaaccagctc ctgcaccatc tacaaacaca 2820
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actaatacaa actcaaatc taatacgaat acaactcaa atacgaatgc taatcaaggt 2940
tcttccaaca ataacagcaa ttcaagtgc agtgctatta ttgctgaagc tcaaaaaac 3000
cttggaacag ctattcatg ggggtgtaac ggaccaacta catttgattg ctctgggtac 3060
actaaatag tatttgctaa agcgggtatc tcccttcac gtacatctg cgacaaatc 3120
gctagcacta caagaatttc tgaattctca gcaaacctg gtgatttagt attcttcgac 3180
tatggtagcg gaatttctc cattggtatt tatgttggt atggcaaat gattaacgcg 3240
caagacaatg gcgttaata cgataacac caccgctctg gctggggtaa atatctagtt 3300
ggcttcggtc gcgtataata aggatcc 3327

```

<210> SEQ ID NO 45
 <211> LENGTH: 26
 <212> TYPE: PRT
 <213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 45

```

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu
 1             5             10             15

```

```

Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp
      20             25

```

<210> SEQ ID NO 46
 <211> LENGTH: 29
 <212> TYPE: PRT
 <213> ORGANISM: *Lactococcus lactis*

<400> SEQUENCE: 46

```

Met Lys Lys Lys Ile Ile Ser Ala Ile Leu Met Ser Thr Val Ile Leu
 1             5             10             15

```

```

Ser Ala Ala Ala Pro Leu Ser Gly Val Tyr Ala Asp Thr
      20             25

```

<210> SEQ ID NO 47
 <211> LENGTH: 31
 <212> TYPE: PRT
 <213> ORGANISM: *Bacillus anthracis*

<400> SEQUENCE: 47

```

Met Lys Lys Arg Lys Val Leu Ile Pro Leu Met Ala Leu Ser Thr Ile
 1             5             10             15

```

-continued

Leu Val Ser Ser Thr Gly Asn Leu Glu Val Ile Gln Ala Glu Val
20 25 30

<210> SEQ ID NO 48
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 48

Met Asn Met Lys Lys Ala Thr Ile Ala Ala Thr Ala Gly Ile Ala Val
1 5 10 15
Thr Ala Phe Ala Ala Pro Thr Ile Ala Ser Ala Ser Thr
20 25

<210> SEQ ID NO 49
<211> LENGTH: 54
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 49

Met Gln Lys Thr Arg Lys Glu Arg Ile Leu Glu Ala Leu Gln Glu Glu
1 5 10 15
Lys Lys Asn Lys Lys Ser Lys Lys Phe Lys Thr Gly Ala Thr Ile Ala
20 25 30
Gly Val Thr Ala Ile Ala Thr Ser Ile Thr Val Pro Gly Ile Glu Val
35 40 45
Ile Val Ser Ala Asp Glu
50

<210> SEQ ID NO 50
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: *Bacillus anthracis*

<400> SEQUENCE: 50

Met Lys Lys Leu Lys Met Ala Ser Cys Ala Leu Val Ala Gly Leu Met
1 5 10 15
Phe Ser Gly Leu Thr Pro Asn Ala Phe Ala Glu Asp
20 25

<210> SEQ ID NO 51
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: *Staphylococcus aureus*

<400> SEQUENCE: 51

Met Ala Lys Lys Phe Asn Tyr Lys Leu Pro Ser Met Val Ala Leu Thr
1 5 10 15
Leu Val Gly Ser Ala Val Thr Ala His Gln Val Gln Ala Ala Glu
20 25 30

<210> SEQ ID NO 52
<211> LENGTH: 59
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 52

Met Thr Asp Lys Lys Ser Glu Asn Gln Thr Glu Lys Thr Glu Thr Lys
1 5 10 15

-continued

Glu Asn Lys Gly Met Thr Arg Arg Glu Met Leu Lys Leu Ser Ala Val
20 25 30

Ala Gly Thr Gly Ile Ala Val Gly Ala Thr Gly Leu Gly Thr Ile Leu
35 40 45

Asn Val Val Asp Gln Val Asp Lys Ala Leu Thr
50 55

<210> SEQ ID NO 53
<211> LENGTH: 53
<212> TYPE: PRT
<213> ORGANISM: *Bacillus subtilis*

<400> SEQUENCE: 53

Met Ala Tyr Asp Ser Arg Phe Asp Glu Trp Val Gln Lys Leu Lys Glu
1 5 10 15

Glu Ser Phe Gln Asn Asn Thr Phe Asp Arg Arg Lys Phe Ile Gln Gly
20 25 30

Ala Gly Lys Ile Ala Gly Leu Ser Leu Gly Leu Thr Ile Ala Gln Ser
35 40 45

Val Gly Ala Phe Gly
50

<210> SEQ ID NO 54
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 54

Thr Glu Ala Lys Asp
1 5

<210> SEQ ID NO 55
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Lactococcus lactis*

<400> SEQUENCE: 55

Val Tyr Ala Asp Thr
1 5

<210> SEQ ID NO 56
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Bacillus anthracis*

<400> SEQUENCE: 56

Ile Gln Ala Glu Val
1 5

<210> SEQ ID NO 57
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 57

Ala Ser Ala Ser Thr
1 5

<210> SEQ ID NO 58

-continued

<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 58

Val Ser Ala Asp Glu
1 5

<210> SEQ ID NO 59
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Bacillus anthracis*

<400> SEQUENCE: 59

Ala Phe Ala Glu Asp
1 5

<210> SEQ ID NO 60
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Staphylococcus aureus*

<400> SEQUENCE: 60

Val Gln Ala Ala Glu
1 5

<210> SEQ ID NO 61
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 61

Asp Lys Ala Leu Thr
1 5

<210> SEQ ID NO 62
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: *Bacillus subtilis*

<400> SEQUENCE: 62

Val Gly Ala Phe Gly
1 5

<210> SEQ ID NO 63
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: *Homo sapiens*

<400> SEQUENCE: 63

Val Leu Gln Glu Leu Asn Val Thr Val
1 5

<210> SEQ ID NO 64
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: *Homo sapiens*

<400> SEQUENCE: 64

Ser Leu Leu Phe Leu Leu Phe Ser Leu
1 5

-continued

<210> SEQ ID NO 65
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 65

Val Leu Pro Leu Thr Val Ala Glu Val
1 5

<210> SEQ ID NO 66
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 66

Glu Leu Ala Val Ala Leu Ala Gln Lys
1 5

<210> SEQ ID NO 67
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 67

Ala Leu Gln Gly Gly Gly Pro Pro Tyr
1 5

<210> SEQ ID NO 68
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 68

Phe Tyr Pro Gly Tyr Leu Cys Ser Leu
1 5

<210> SEQ ID NO 69
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 69

Leu Tyr Pro Lys Ala Arg Leu Ala Phe
1 5

<210> SEQ ID NO 70
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Listeria monocytogenes

<400> SEQUENCE: 70

aaggagagtg aaacccatg

19

<210> SEQ ID NO 71
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Listeria monocytogenes

<400> SEQUENCE: 71

taaaaacaca gaacgaaga aaaagtgagg tgaatga

37

<210> SEQ ID NO 72

-continued

<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 72

Ser Pro Ser Tyr Val Tyr His Gln Phe
1 5

<210> SEQ ID NO 73
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Altered epitope

<400> SEQUENCE: 73

Ser Pro Ser Tyr Ala Tyr His Gln Phe
1 5

<210> SEQ ID NO 74
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 74

ctctggtacc tcctttgatt agtatattc

29

<210> SEQ ID NO 75
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 75

caatgcatcc ctcgagatca taatttactt catccc

36

<210> SEQ ID NO 76
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 76

atttctcgag tccatggggg gttctcatca tc

32

<210> SEQ ID NO 77
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 77

ggtgctcgag tgcggccgca agctt

25

<210> SEQ ID NO 78
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Synthetic oligonucleotide

<400> SEQUENCE: 78

cgattcccct agttatgttt accaccaatt tgctgca 37

<210> SEQ ID NO 79

<211> LENGTH: 31

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic oligonucleotide

<400> SEQUENCE: 79

gcaaattggt ggtaaacata actaggggaa t 31

<210> SEQ ID NO 80

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Coding sequence of altered epitope

<400> SEQUENCE: 80

agtcceaagtt atgcatatca tcaattt 27

<210> SEQ ID NO 81

<211> LENGTH: 33

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic oligonucleotide

<400> SEQUENCE: 81

cgatagtcca agttatgcat atcatcaatt tgc 33

<210> SEQ ID NO 82

<211> LENGTH: 34

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic oligonucleotide

<400> SEQUENCE: 82

gtcgcacaatt gatgatatgc ataacttgga ctat 34

<210> SEQ ID NO 83

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 83

cccttgggga tccttaatta tacg 24

<210> SEQ ID NO 84

<211> LENGTH: 29

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 84

-continued

ctctggatcc atccgcgtgt ttcttttcg 29

<210> SEQ ID NO 85
<211> LENGTH: 16
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Consensus sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 1, 9, 10, 11, 12, 13
<223> OTHER INFORMATION: n = A,U,C or G

<400> SEQUENCE: 85

naggagggunn nnnaug 16

<210> SEQ ID NO 86
<211> LENGTH: 87
<212> TYPE: DNA
<213> ORGANISM: Lactococcus lactis

<400> SEQUENCE: 86

atgaaaaaaa agattatctc agctatttta atgtctacag tgatactttc tgctgcagcc 60

ccgttgctcag gtgttttacgc tgacaca 87

<210> SEQ ID NO 87
<211> LENGTH: 87
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 87

atgaaaaaaa aaattattag tgcaatttta atgagtacag ttattttaag tgcagcagca 60

ccattaagtg gtgttttatgc agataca 87

<210> SEQ ID NO 88
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 88

aaggagagtg aaacccatga atatgaaaa agcaac 36

<210> SEQ ID NO 89
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 89

gtgtgatgga tatctgcaga attc 24

<210> SEQ ID NO 90
<211> LENGTH: 87
<212> TYPE: DNA
<213> ORGANISM: Listeria monocytogenes

<400> SEQUENCE: 90

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atgaatatga aaaaagcaac tatcgcggt acagctggga ttgcggtaac agcatttgct 60
gcgccaacaa tcgcatccgc aagcact 87

<210> SEQ ID NO 91
<211> LENGTH: 87
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 91

atgaatatga aaaaagcaac aattgcagca acagcaggta ttgcagttac agcatttgca 60
gcaccaacaa ttgcaagtgc aagtaca 87

<210> SEQ ID NO 92
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 92

gacgtcaata cgactcacta tag 23

<210> SEQ ID NO 93
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 93

cttttttcat attcatgggt ttactctcc ttctac 36

<210> SEQ ID NO 94
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 94

gtcaaaacat acgctcttat c 21

<210> SEQ ID NO 95
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 95

acataatcag tccaaagtag atgc 24

<210> SEQ ID NO 96
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 96

-continued

gattataaag atgatgatga taaa

24

<210> SEQ ID NO 97
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic epitope tag

<400> SEQUENCE: 97

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

<210> SEQ ID NO 98
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 98

gaacaaaaat taattagtga agaagattta

30

<210> SEQ ID NO 99
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 99

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
1 5 10

<210> SEQ ID NO 100
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Listeria monocytogenes

<400> SEQUENCE: 100

Asn Glu Lys Tyr Ala Gln Ala Tyr Pro Asn Val Ser
1 5 10

<210> SEQ ID NO 101
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Listeria monocytogenes

<400> SEQUENCE: 101

Val Ala Tyr Gly Arg Gln Val Tyr Leu
1 5

<210> SEQ ID NO 102
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Listeria monocytogenes

<400> SEQUENCE: 102

Gly Tyr Lys Asp Gly Asn Glu Tyr Ile
1 5

<210> SEQ ID NO 103
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 103

gttaagtttc atgtggacgg caaag 25

<210> SEQ ID NO 104
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 104

aggtcttttt cagttaacta tcctctcctt gattctagtt at 42

<210> SEQ ID NO 105
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 105

caaggagagg atagttaact gaaaaagacc taaaaaagaa ggc 43

<210> SEQ ID NO 106
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 106

tccctgttc ctataattgt tagtc 26

<210> SEQ ID NO 107
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 107

gtggacggca aagaaacaac caaag 25

<210> SEQ ID NO 108
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 108

gttcctataa ttgtagctc attttttc 29

<210> SEQ ID NO 109
<211> LENGTH: 59
<212> TYPE: PRT
<213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 109

Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu

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1	5	10	15
Pro Ile Ala Gln Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys			
20	25	30	
Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser			
35	40	45	
Pro Lys Thr Pro Ile Glu Lys Lys His Ala Asp			
50	55		

<210> SEQ ID NO 110
 <211> LENGTH: 177
 <212> TYPE: DNA
 <213> ORGANISM: *Listeria monocytogenes*

<400> SEQUENCE: 110

atgaaaaaaa taatgctagt ttttattaca cttatattag ttagtctacc aattgcgcaa	60
caaaactgaag caaaggatgc atctgcattc aataaagaaa attcaatttc atccatggca	120
ccaccagcat ctccgcctgc aagtcctaag acgccaatcg aaaagaaaca cgcggtat	177

<210> SEQ ID NO 111
 <211> LENGTH: 93
 <212> TYPE: DNA
 <213> ORGANISM: *Bacillus anthracis*

<400> SEQUENCE: 111

atgaaaaaac gaaaagtgtt aataccatta atggcattgt ctacgatatt agtttcaagc	60
acaggteatt tagaggtgat tcaggcagaa gtt	93

<210> SEQ ID NO 112
 <211> LENGTH: 156
 <212> TYPE: DNA
 <213> ORGANISM: *Bacillus subtilis*

<400> SEQUENCE: 112

atggcatatcg acagtctgtt tgatgaatgg gtacagaaac tgaaagagga aagctttcaa	60
aacaatacgt ttgaccgccg caaatattatt caaggagcgg ggaagattgc aggactttct	120
cttgatttaa cgattgccca gtcggttggg gccttt	156

<210> SEQ ID NO 113
 <211> LENGTH: 177
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 113

atgaaaaaaa ttatgttagt ttttattaca ttaatttttag ttagtttacc aattgcacaa	60
caaacagaag caaaagatgc aagtgcattt aataaagaaa atagtattag tagtatggca	120
ccaccagcaa gtccaccagc aagtccaaaa acaccaattg aaaaaaaca tgcagat	177

<210> SEQ ID NO 114
 <211> LENGTH: 93
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Codon-optimized sequence

<400> SEQUENCE: 114

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```

atgaaaaaac gtaaagtttt aattccatta atggcattaa gtacaatttt agtttagtagt    60
acaggtaatt tagaagttat tcaagcagaa gtt                                93

```

```

<210> SEQ ID NO 115
<211> LENGTH: 156
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon-optimized sequence

```

```

<400> SEQUENCE: 115
atggcatatg atagtcgttt tgatgaatgg gttcaaaaat taaaagaaga aagttttcaa    60
aataatacat ttgatcgctg taaatttatt caaggtgcag gtaaaattgc aggtttaagt    120
ttaggtttaa caattgcaca aagtgttggt gcattt                                156

```

```

<210> SEQ ID NO 116
<211> LENGTH: 70
<212> TYPE: PRT
<213> ORGANISM: Listeria monocytogenes

```

```

<400> SEQUENCE: 116
Met Asn Met Lys Lys Ala Thr Ile Ala Ala Thr Ala Gly Ile Ala Val
 1             5             10            15
Thr Ala Phe Ala Ala Pro Thr Ile Ala Ser Ala Ser Thr Val Val Val
      20            25            30
Glu Ala Gly Asp Thr Leu Trp Gly Ile Ala Gln Ser Lys Gly Thr Thr
      35            40            45
Val Asp Ala Ile Lys Lys Ala Asn Asn Leu Thr Thr Asp Lys Ile Val
      50            55            60
Pro Gly Gln Lys Leu Gln
65              70

```

```

<210> SEQ ID NO 117
<211> LENGTH: 28
<212> TYPE: DNA
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naggaggunn nnnnnnnau g 21

We claim:

1. A recombinant nucleic acid molecule, comprising:
 - (a) a first polynucleotide encoding a signal peptide native to a bacterium, wherein the first polynucleotide is codon-optimized for expression in the bacterium; and
 - (b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide,

wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide.

2. The recombinant nucleic acid molecule of claim 1, wherein the bacterium is a *Listeria* bacterium.

3. The recombinant nucleic acid molecule of claim 1, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of a tumor-associated antigen, a polypeptide derived from a tumor-associated antigen, an infectious disease antigen, and a polypeptide derived from an infectious disease antigen.

4. The recombinant nucleic acid molecule of claim 1, wherein the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide, foreign to the bacterium, or both.

5. The recombinant nucleic acid molecule of claim 1, wherein the signal peptide is an LLO signal peptide from *Listeria monocytogenes* or is a p60 signal peptide from *Listeria monocytogenes*.

6. An expression cassette comprising the recombinant nucleic acid molecule of claim 1, further comprising a promoter operably linked to the first and second polynucleotides of the recombinant nucleic acid molecule.

7. A recombinant bacterium comprising the recombinant nucleic acid molecule of claim 1, wherein the first polynucleotide is codon-optimized for expression in the recombinant bacterium.

8. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium of claim 7, wherein the polypeptide encoded by the second polynucleotide comprises the antigen.

9. A recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:

(a) a first polynucleotide encoding a signal peptide, wherein the first polynucleotide is codon-optimized for expression in a *Listeria* bacterium; and

(b) a second polynucleotide encoding a polypeptide, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide,

wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide.

10. The recombinant *Listeria* bacterium of claim 9, which comprises an expression cassette comprising the recombinant nucleic acid molecule, wherein the expression cassette further comprises a promoter operably linked to both the first and second polynucleotides of the recombinant nucleic acid molecule.

11. The recombinant *Listeria* bacterium of claim 9, wherein the *Listeria* bacterium belongs to the species *Listeria monocytogenes*.

12. The recombinant *Listeria* bacterium of claim 9, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of a tumor-associated antigen, a polypeptide derived from a tumor-associated antigen, an infectious disease antigen, and a polypeptide derived from an infectious disease antigen.

13. The recombinant *Listeria* bacterium of claim 12, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or comprises a polypeptide derived from an antigen selected from the group

consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA.

14. The recombinant *Listeria* bacterium of claim 13, wherein the polypeptide encoded by the second polynucleotide comprises mesothelin, or an antigenic fragment or antigenic variant thereof, or comprises NY-ESO-1, or an antigenic fragment or antigenic variant thereof.

15. The recombinant *Listeria* bacterium of claim 14, wherein the polypeptide encoded by the second polynucleotide comprises human mesothelin deleted of its signal peptide and GPI linker domain.

16. The recombinant *Listeria* bacterium of claim 9, wherein the polypeptide encoded by the second polynucleotide is heterologous to the signal peptide.

17. The recombinant *Listeria* bacterium of claim 9, wherein the signal peptide is foreign to the *Listeria* bacterium.

18. The recombinant *Listeria* bacterium of claim 9, wherein the signal peptide is native to the *Listeria* bacterium.

19. The recombinant *Listeria* bacterium of claim 9, wherein the signal peptide is a signal peptide selected from the group consisting of an LLO signal peptide from *Listeria monocytogenes*, a Usp45 signal peptide from *Lactococcus lactis*, a Protective Antigen signal peptide from *Bacillus anthracis*, a p60 signal peptide from *Listeria monocytogenes*, and a PhoD signal peptide from *B. subtilis*.

20. The recombinant *Listeria* bacterium of claim 9, which is attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation.

21. The recombinant *Listeria* bacterium of claim 9, which is deficient with respect to ActA, Internalin B, or both ActA and Internalin B.

22. The recombinant *Listeria* bacterium of claim 9, wherein the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound.

23. An immunogenic composition or vaccine comprising the recombinant *Listeria* bacterium of claim 9.

24. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 9, wherein the polypeptide encoded by the second polynucleotide comprises the antigen.

25. A method of preventing or treating a condition in a host comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 9.

26. A recombinant nucleic acid molecule, comprising:

(a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; and

(b) a second polynucleotide encoding a polypeptide heterologous to the signal peptide,

wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide, and

wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide.

27. The recombinant nucleic acid molecule of claim 26, wherein the first polynucleotide, the second polynucleotide,

or both the first and second polynucleotides are codon-optimized for expression in a bacterium.

28. The recombinant nucleic acid molecule of claim 26, wherein the signal peptide is a Listerial signal peptide.

29. The recombinant nucleic acid molecule of claim 26, wherein the signal peptide is a secA2 signal peptide or a Tat signal peptide.

30. The recombinant nucleic acid molecule of claim 26, wherein the signal peptide is a p60 signal peptide from *Listeria monocytogenes* or is a phoD signal peptide from *B. subtilis*.

31. The recombinant nucleic acid molecule of claim 26, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of a tumor-associated antigen, a polypeptide derived from a tumor-associated antigen, an infectious disease antigen, and a polypeptide derived from an infectious disease antigen.

32. An expression cassette comprising the recombinant nucleic acid molecule of claim 26, further comprising a promoter operably linked to the first and second polynucleotides of the recombinant nucleic acid molecule.

33. A recombinant bacterium comprising the recombinant nucleic acid molecule of claim 26.

34. The recombinant bacterium of claim 33, which is a *Listeria* bacterium.

35. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium of claim 33, wherein the polypeptide encoded by the second polynucleotide comprises the antigen.

36. A recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:

- (a) a first polynucleotide encoding a non-secA1 bacterial signal peptide; and
- (b) a second polynucleotide encoding a polypeptide which is heterologous to the signal peptide or is foreign to the bacterium, or both, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide;

wherein the recombinant nucleic acid molecule encodes a fusion protein comprising the signal peptide and the polypeptide.

37. The recombinant *Listeria* bacterium of claim 36, which comprises an expression cassette comprising the recombinant nucleic acid molecule, wherein the expression cassette further comprises a promoter operably linked to both the first and second polynucleotides of the recombinant nucleic acid molecule.

38. The recombinant *Listeria* bacterium of claim 36, wherein the first polynucleotide, the second polynucleotide, or both the first and second polynucleotide are codon-optimized for expression in the *Listeria* bacterium.

39. The recombinant *Listeria* bacterium of claim 36, wherein the bacterium belongs to the species *Listeria monocytogenes*.

40. The recombinant *Listeria* bacterium of claim 36, wherein the non-secA1 signal peptide is a non-Listerial signal peptide.

41. The recombinant *Listeria* bacterium of claim 36, wherein the non-secA1 signal peptide is a Listerial signal peptide.

42. The recombinant *Listeria* bacterium of claim 36, wherein the signal peptide is a secA2 signal peptide.

43. The recombinant *Listeria* of claim 42, wherein the recombinant nucleic acid molecule further comprises:

- (c) a third polynucleotide encoding a secA2 autolysin, or a fragment thereof, in the same translational reading frame as the first and second polynucleotides, wherein the second polynucleotide is positioned within the third polynucleotide or between the first and third polynucleotides.

44. The recombinant *Listeria* bacterium of claim 36, wherein the signal peptide is a Tat signal peptide.

45. The recombinant *Listeria* bacterium of claim 36, wherein the signal peptide is a PhoD signal peptide from *B. subtilis* or is a p60 signal peptide from *Listeria monocytogenes*.

46. The recombinant *Listeria* bacterium of claim 36, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of a tumor-associated antigen, a polypeptide derived from a tumor-associated antigen, an infectious disease antigen, and a polypeptide derived from an infectious disease antigen.

47. The recombinant *Listeria* bacterium of claim 46, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or comprises a polypeptide derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA.

48. The recombinant *Listeria* bacterium of claim 47, wherein the polypeptide encoded by the second polynucleotide comprises mesothelin, or an antigenic fragment or antigenic variant thereof.

49. The recombinant *Listeria* bacterium of claim 48, wherein the polypeptide encoded by the second polynucleotide comprises human mesothelin deleted of its signal peptide and GPI anchor.

50. The recombinant *Listeria* bacterium of claim 36, which is attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation.

51. The recombinant *Listeria* bacterium of claim 36, which is deficient with respect to ActA, Internalin B, or both ActA and Internalin B.

52. The recombinant *Listeria* bacterium of claim 36, wherein the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound.

53. An immunogenic composition or vaccine comprising the recombinant *Listeria* bacterium of claim 36.

54. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 36, wherein the polypeptide encoded by the second polynucleotide comprises the antigen.

55. A method of preventing or treating a condition in a host comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 36.

56. A recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:

- (a) a first polynucleotide encoding a non-Listerial signal peptide; and
- (b) a second polynucleotide encoding a polypeptide that is in the same translational reading frame as the first polynucleotide,

wherein the recombinant nucleic acid molecule encodes a fusion protein comprising both the non-Listerial signal peptide and the polypeptide.

57. The recombinant *Listeria* bacterium of claim 56, which comprises an expression cassette comprising the recombinant nucleic acid molecule, wherein the expression cassette further comprises a promoter operably linked to both the first and second polynucleotides of the recombinant nucleic acid molecule.

58. The recombinant *Listeria* bacterium of claim 56, wherein the first polynucleotide, the second polynucleotide, or both the first and second polynucleotides are codon-optimized for expression in the *Listeria* bacterium.

59. The recombinant *Listeria* bacterium of claim 56, wherein the bacterium is *Listeria monocytogenes*.

60. The recombinant *Listeria* bacterium of claim 56, wherein the signal peptide is bacterial.

61. The recombinant *Listeria* bacterium of claim 60, wherein the signal peptide is derived from a gram positive bacterium.

62. The recombinant *Listeria* bacterium of claim 61, wherein the signal peptide is derived from a bacterium belonging to the genus *Bacillus*, *Staphylococcus*, or *Lactococcus*.

63. The recombinant *Listeria* bacterium of claim 62, wherein the signal peptide is a signal peptide selected from the group consisting of a Usp45 signal peptide from *Lactococcus lactis*, a Protective Antigen signal peptide from *Bacillus anthracis*, and a PhoD signal peptide from *Bacillus subtilis*.

64. The recombinant *Listeria* bacterium of claim 56, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of a tumor-associated antigen, a polypeptide derived from a tumor-associated antigen, an infectious disease antigen, and a polypeptide derived from an infectious disease antigen.

65. The recombinant *Listeria* bacterium of claim 64, wherein the polypeptide encoded by the second polynucleotide comprises an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA, or comprises a polypeptide derived from an antigen selected from the group consisting of K-Ras, H-Ras, N-Ras, 12-K-Ras, mesothelin, PSCA, NY-ESO-1, WT-1, survivin, gp100, PAP, proteinase 3, SPAS-1, SP-17, PAGE-4, TARP, and CEA.

66. The recombinant *Listeria* bacterium of claim 65, wherein the polypeptide encoded by the second polynucleotide comprises mesothelin, or an antigenic fragment or antigenic variant thereof.

67. The recombinant *Listeria* bacterium of claim 66, wherein the polypeptide encoded by the second polynucleotide comprises human mesothelin deleted of its signal peptide and GPI anchor.

68. The recombinant *Listeria* bacterium of claim 56, which is attenuated for cell-to-cell spread, entry into non-phagocytic cells, or proliferation.

69. The recombinant *Listeria* bacterium of claim 56, which is deficient with respect to ActA, Internalin B, or both ActA and Internalin B.

70. The recombinant *Listeria* bacterium of claim 56, wherein the nucleic acid of the recombinant bacterium has been modified by reaction with a nucleic acid targeting compound.

71. An immunogenic composition or vaccine comprising the recombinant *Listeria* bacterium of claim 56.

72. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 56, wherein the polypeptide encoded by the second polynucleotide comprises the antigen.

73. A method of preventing or treating a condition in a host comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 56.

74. A recombinant nucleic acid molecule, comprising:

- (a) a first polynucleotide encoding a bacterial autolysin, or a catalytically active fragment or catalytically active variant thereof; and
- (b) a second polynucleotide encoding a polypeptide heterologous to the bacterial autolysin, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide,

wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the polypeptide encoded by the second polynucleotide and the autolysin, or catalytically active fragment or catalytically active variant thereof, wherein in the protein chimera the polypeptide is fused to the autolysin, or catalytically active fragment or catalytically active variant thereof, or is positioned within the autolysin, or catalytically active fragment or catalytically active variant thereof.

75. A recombinant bacterium comprising the recombinant nucleic acid molecule of claim 74.

76. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium of claim 75, wherein the polypeptide encoded by the second polynucleotide comprises the antigen.

77. A recombinant *Listeria* bacterium comprising a polycistronic expression cassette, wherein the polycistronic expression cassette encodes at least two discrete non-Listerial polypeptides.

78. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 77, wherein at least one of the non-Listerial polypeptides encoded by the polycistronic expression cassette comprises the antigen.

79. A recombinant *Listeria* bacterium comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a polynucleotide encoding a polypeptide foreign to the *Listeria* bacterium, wherein the polynucleotide is codon-optimized for expression in *Listeria*.

80. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant *Listeria* bacterium of claim 79, wherein the foreign polypeptide comprises the antigen.

81. A recombinant nucleic acid molecule, comprising:

- (a) a first polynucleotide encoding a signal peptide;
- (b) a second polynucleotide encoding a secreted protein, or a fragment thereof, wherein the second polynucleotide is in the same translational reading frame as the first polynucleotide; and
- (c) a third polynucleotide encoding a polypeptide heterologous to the secreted protein, or fragment thereof,

wherein the third polynucleotide is in the same translational reading frame as the first and second polynucleotides,

wherein the recombinant nucleic acid molecule encodes a protein chimera comprising the signal peptide, the polypeptide encoded by the third polynucleotide, and the secreted protein, or fragment thereof, and wherein the polypeptide encoded by the third polynucleotide is fused to the secreted protein, or fragment thereof, or is positioned within the secreted protein, or fragment thereof, in the protein chimera.

82. A recombinant bacterium comprising the recombinant nucleic acid molecule of claim 81.

83. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the recombinant bacterium of claim 82, wherein the polypeptide encoded by the third polynucleotide comprises the antigen.

* * * * *

EXHIBIT 6

CERUS

CRS-207: Live-Attenuated *Listeria* monocytogenes Encoding Mesothelin for Immunotherapy of Patients with Pancreatic and Ovarian Cancers

D.G. Brockstedt¹, M.L. Leong¹, K.S. Bahjat¹, W. Liu¹, M.A.
Giedlin¹, R.A. Prell¹, E.E. Lemmens¹, B. Hanson¹, P. Lauer¹, T.C.
Wu², C.F. Hung², D. Laheru², E.M. Jaffee², D.M. Pardoll², J.
Eiden¹, and T.W. Dubensky¹

¹Cerus Corporation, Concord, CA, ² Johns Hopkins University,
Baltimore, MD

Abstract

CRS-207 is a live-attenuated *Listeria monocytogenes* (*Lm*) vaccine strain encoding human Mesothelin (*Lm* $\Delta actA/\Delta inlB$ -hMeso) that is being developed by Cerus Corporation for clinical evaluation in subjects with pancreatic and ovarian carcinoma. Mesothelin is a tumor-associated antigen that is broadly expressed across pancreas and ovarian cancers, but not in normal cells from these organs. In early-phase clinical trials conducted in patients with pancreatic carcinoma, Mesothelin has been shown to be a promising immune target against which vaccine-induced effector T cells have been correlated with patient benefit (Jaffee et. al., 2001, and Thomas et. al., 2004). Expression of Mesothelin in CRS-207 was developed from the *Lm* platform strain CRS-100, which has engineered deletions of two genes encoding the virulence determinants *actA* and internalin B (*inlB*; *Lm* $\Delta actA/\Delta inlB$). CRS-100 is an investigational agent currently undergoing clinical evaluation in a Phase 1 dose-escalation study of safety and tolerability following intravenous administration in adults with carcinoma and liver metastases (VAC05001).

Introduction and Approach

Mesothelin

- Positive clinical outcomes observed in patients with pancreatic carcinoma after vaccination with allogeneic whole cell vaccine encoding GM-CSF (Jaffee et. al. (2001) *JCO*, 19:145-156, and Thomas et. al. (2004) *J. Exp. Med.* 200:297-306)
 - » Tumor-specific DTH response observed in 3 of 8 patients given high-dose vaccine ($\geq 1 \times 10^8$ cells)
 - » All three alive and tumor-free greater than 7 years post-diagnosis
 - » Mesothelin-specific T cell responses only in long-term survivors
 - » Responses seen across HLA haplotypes (A2, A3 and A24)
 - » Mesothelin-specific T cells kill human tumors *in vitro*
 - » No autoimmunity observed
 - » Mesothelin is a validated immune target for pancreatic carcinoma

Listeria monocytogenes (Lm)

- *Lm* is an attractive vector platform due to potent vaccine-induced innate immunity and acquired CD4+/CD8+ T-cell immunity specific for encoded heterologous antigens
- *Lm* is a ubiquitous, food-borne pathogen to which most adults have been exposed
- We have developed *Lm* $\Delta actA/\Delta inlB$, a live-attenuated vaccine platform strain deleted of two virulence determinants that is 1000-fold attenuated compare to wild-type *Lm* (Brockstedt et al, 2004)
- CRS-207 is a recombinant *Lm* $\Delta actA/\Delta inlB$ vaccine strain that contains a human Mesothelin expression cassette integrated at a specific location in the bacterial chromosome sequence.

Mesothelin is a Validated Immune Target for Pancreatic Carcinoma

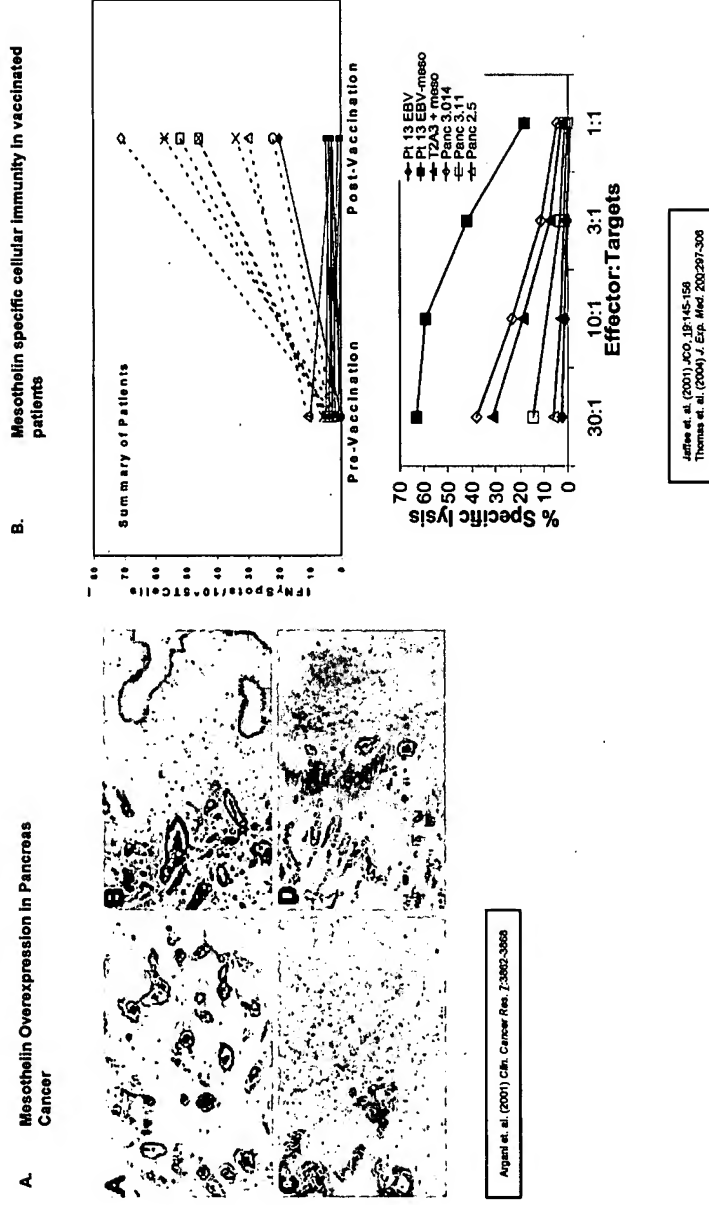
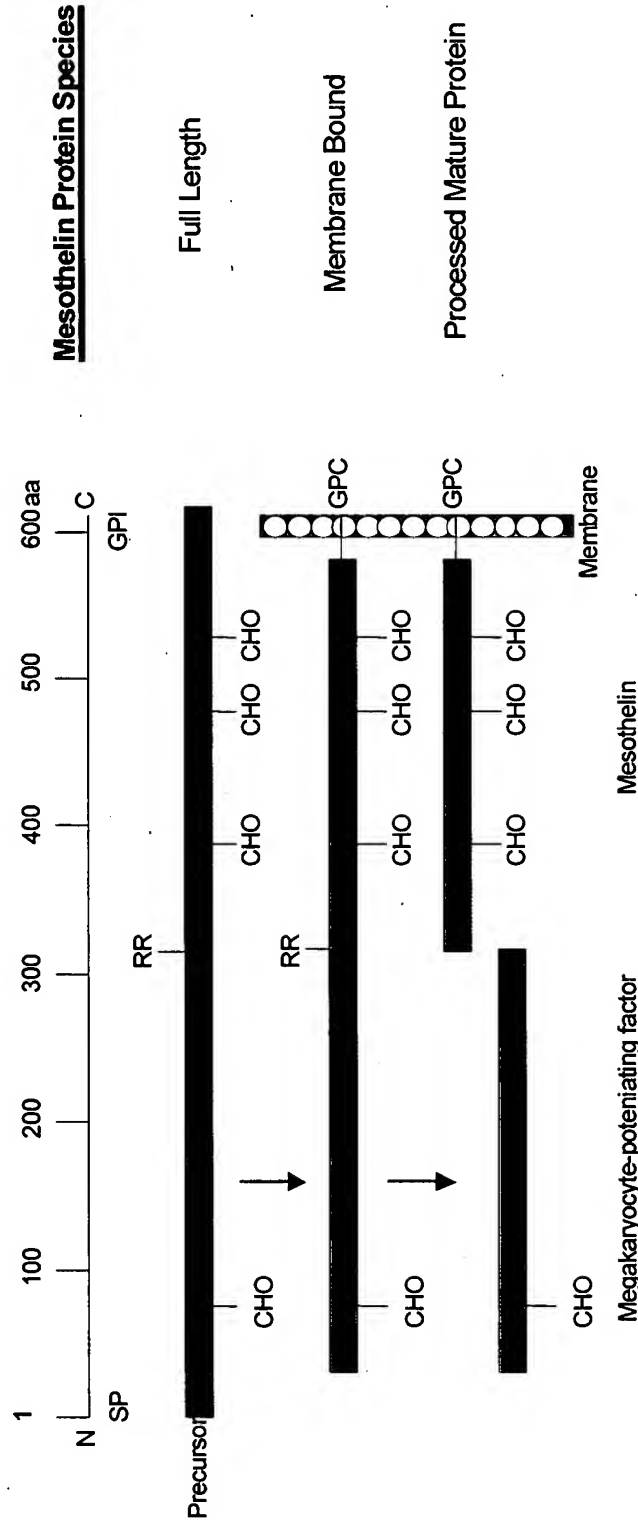


Figure 1. (A) Mesothelin is broadly overexpressed in pancreatic carcinoma. Immunohistochemistry for Mesothelin. (A-C) Strong labeling of the neoplastic epithelium and a complete absence of labeling in the non-neoplastic epithelium and stroma. (D) Luminal accentuation of Mesothelin labeling. In this study, Mesothelin RNA was detected in 100% (60/60) unique primary ductal pancreatic adenocarcinoma tissues, by RT-PCR (from Argani et al., 2001). **(B) Panel 1. Positive DTH responders have Mesothelin-specific cellular immunity.** ELISPOT analysis of CD8+ T cells from PBMCs demonstrates post vaccination induction of mesothelin-specific T cells in three DTH responders. ELISPOT analysis of PBLs from all 14 patients who were treated with the vaccine (Jaffe et al., 2001). ELISPOT analysis for IFN-γ expressing cells was performed using PBMCs that were isolated on the day before vaccination or 28 d after the first vaccination. T2-A2, A3, or A24 cells were pulsed with two mesothelin-derived epitopes or with HIVgag (not shown). All DTH responders are represented by dotted lines and open symbols, and DTH nonresponders are represented by solid lines and closed symbols. Responses above nonspecific background are shown. The number of human IFN-γ spots per 10⁵ CD8+ T cells is plotted.

(B) Panel 2. A Mesothelin-specific CTL line lyses mesothelin-expressing tumor cells. A mesothelin-specific CTL line derived from patient 13 PBL lyses HLA-A3, Mesothelin-expressing cells. Patient-derived CD8+ T cells stimulated with an HLA-A3 mesothelin peptide were tested for their capability to recognize and kill mesothelin-expressing tumor and EBV ⁵¹Cr-labeled target cells. Percent lysis was calculated after 4 h at 37 °C. Results are expressed as the percentage of specific lysis of triplicate samples.

Processing and Maturation of Human Mesothelin



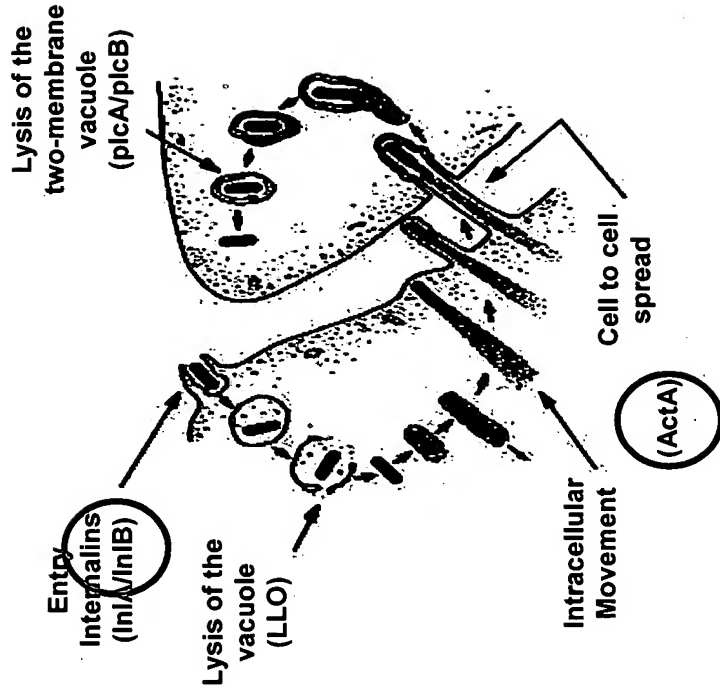
Adopted from Hassan et. al., 2004. Clin. Cancer. Res

Figure 2. Mesothelin biology and biochemistry. Mesothelin is a 40 kDa transmembrane glycoprotein that is expressed primarily in the pleural lining (mesothelium), and at low levels in trachea, tonsil, fallopian tube, and kidney but not in other normal tissue, including pancreas and ovary. Mesothelin may be involved in cell adhesion and tumor metastasis. Mesothelin binds cell surface CA-125, and this interaction effects cell migration in in vitro culture systems. The precursor protein for Mesothelin is synthesized as a 622-amino acid polypeptide with a calculated molecular mass of 77kDa. The potential signal peptide (SP) and the glycosylphosphatidylinositol anchor signal sequence (GASS) are predicted at the amino terminus and the carboxyl terminus, respectively. The precursor protein has four predicted glycosylation sites (CHO) and a furin cleavage site (RR). Cleavage at the furin site generates membrane-bound Mesothelin (green) and the secretory protein, megakaryocyte potentiating factor (red).

Lm $\Delta actA/\Delta inB$ Vaccine Platform Strain is 1000-Fold Attenuated in Mice

CRS-100

A.L. monocytogenes lifecycle and attenuated vaccine platform strain



B. Intracellular infection phenotype of *Lm* $\Delta actA/\Delta inB$ vaccine strain

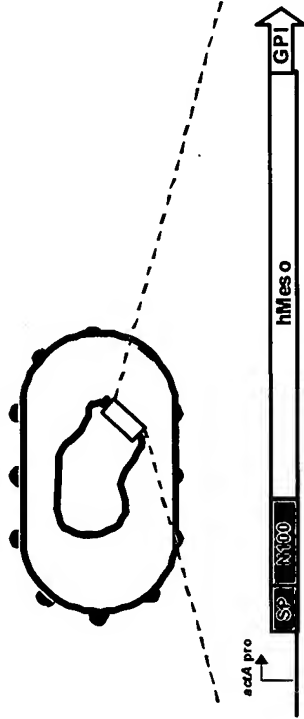
Lm wild-type

Lm $\Delta actA/\Delta inB$

Figure 3. *Lm* vaccine platform strain. (A) *Lm* intracellular lifecycle and attenuation. Schematic representation of *Lm* Internalin B (InlB) mediated infection of hepatocytes via the hepatocyte growth factor receptor (HGFR), vacuolar endocytosis, LLO-mediated escape into the cytosol, bacterial propagation, and ActA-mediated cytosolic motility and spread to neighboring host cells. The *Lm* vaccine platform strain is deleted of the *actA* and *inB* genes, and as a result cannot infect non-phagocytic cells via the HGFR and cannot spread from cell-to-cell. *Lm* $\Delta actA/\Delta inB$ is 1000-fold attenuated in the mouse listeriosis model (Brockstedt et al., 2004. *PNAS*, 101:13832-13837). We have termed this strain CRS-100. **(B)** *Lm* $\Delta actA/\Delta inB$ vaccine platform strain propagates in cytoplasm of infected macrophages but does not spread. J774 mouse macrophage cells were grown on coverslips and infected at an MOI of 5 for 30 min at 37°C. Following washing, infected cells were incubated for 5 hrs at 37°C in the presence of gentamicin. Cells were fixed with 3.5% formaldehyde, stained, and visualized by fluorescent microscopy. Listeria: Rabbit a-Listeria Ab-FITC (green); polymerized host cell Actin: phalloidin-Rhodamine (red); Nucleus: DAPI (blue).

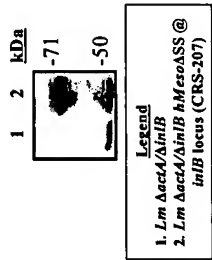
CRS-207 Elicits Human Mesothelin-Specific Cellular Immunity in Vaccinated Mice

A. CRS-207 Schematic



Expression Cassette	
Element	Composition
Promoter	prfA-dependent actA promoter
Signal peptide/fusion partner	ActA 100 N-terminal amino acids
Tumor antigen	Signal peptide-deleted human Mesothelin codon-optimized for expression in <i>Lm</i>

B. Mesothelin expression in CRS-207 infected Macrophages



C. CRS-207 Immunogenicity in Mice

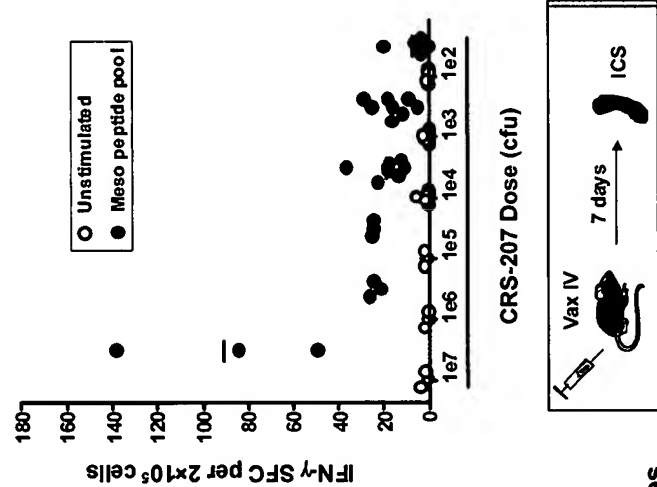


Figure 4. CRS-207 construction and immunogenicity. (A) CRS-207 Schematic. The human Mesothelin expression cassette is integrated in the bacterial chromosome at the inlB locus of the CRS-100 vaccine platform strain. Expression of the ActA N100-Mesothelin (codon-optimized for expression in *Lm*) fusion protein is driven from the PrfA-dependent actA promoter. (B) **Mesothelin expression in CRS-207 infected Macrophages.** Expression and secretion of Mesothelin in J774 mouse macrophage cells infected with CRS-207 was evaluated by Western blot analysis of PAGE-fractionated cell lysates at 8 hrs post infection, using a rabbit polyclonal antibody specific for the ActA 100 N-terminal amino acid residues of the ActA N100-Mesothelin fusion protein. (C) **CRS-207 Immunogenicity in Mice.** Balb/C mice were immunized intravenously (IV) with 5 x 10⁶ colony forming units (cfu) of CRS-207 and splenic Mesothelin-specific cellular immunity was determined 1 week later by ELISPOT assay, using a Mesothelin peptide library that was comprised of 15-mer peptides offset by 4 amino acids covering the entire length of the protein, as stimulators.

CRS-207 Induces Therapeutic Anti-Tumor Responses in Tumor-Bearing Mice

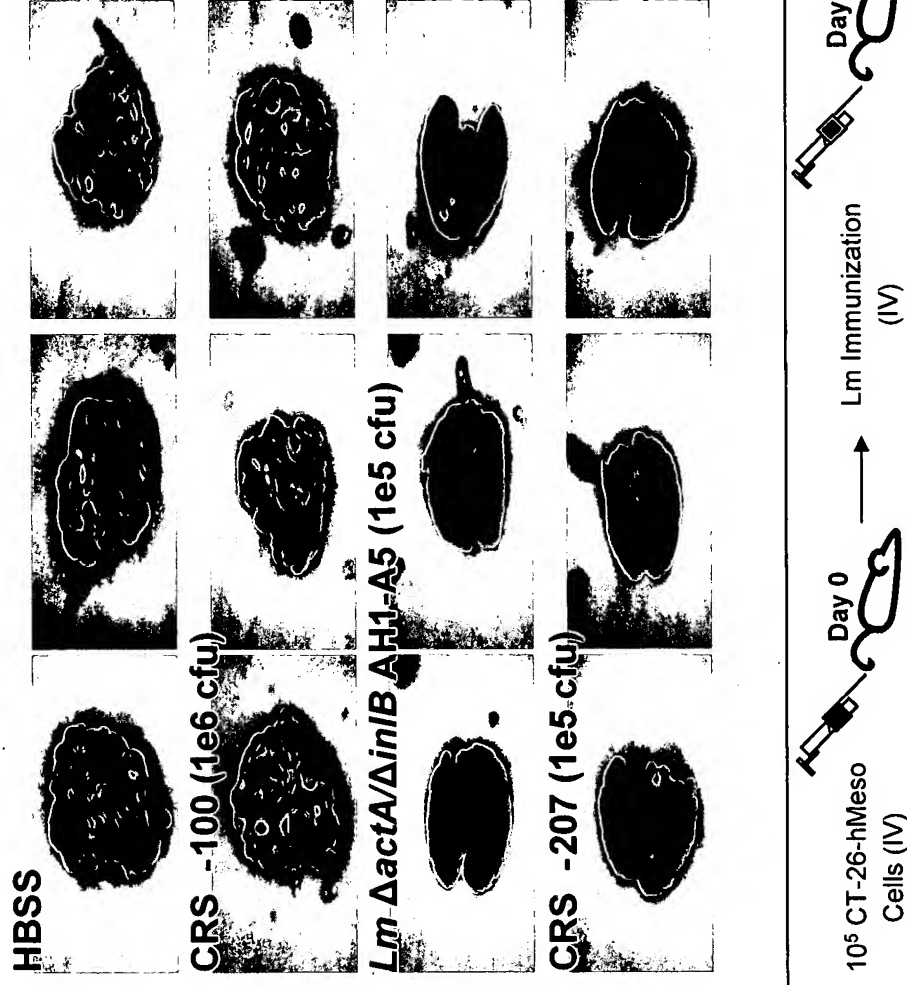


Figure 5. CRS-207 anti-tumor efficacy in mice. Female Balb/c mice were implanted IV with 2×10^5 CT26-Mesothelin tumor cells on day 0. Three days later mice were vaccinated with varying doses of the indicated strain or vehicle control. The *Lm* AH1/A5 vaccine served as a positive control. AH1-A5 is an altered peptide ligand for the L^d immunodominant epitope of the gp70 endogenous rejection antigen expressed by the CT26 adenocarcinoma cell line. Shown are representative lungs 21 days following tumor implantation.

CRS-207 Anti-Tumor Efficacy is Due to Innate and Mesothelin-Specific Cellular Immunity

B. Immune correlates of survival

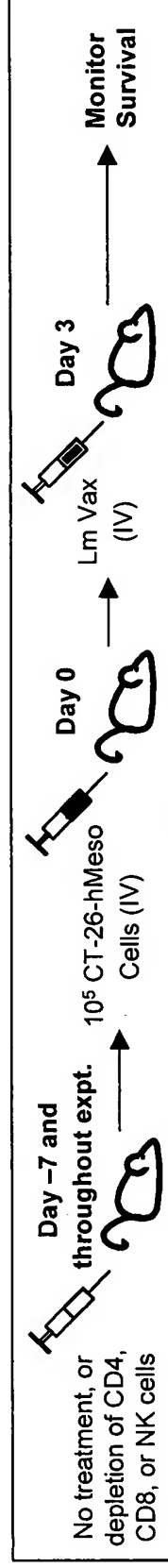
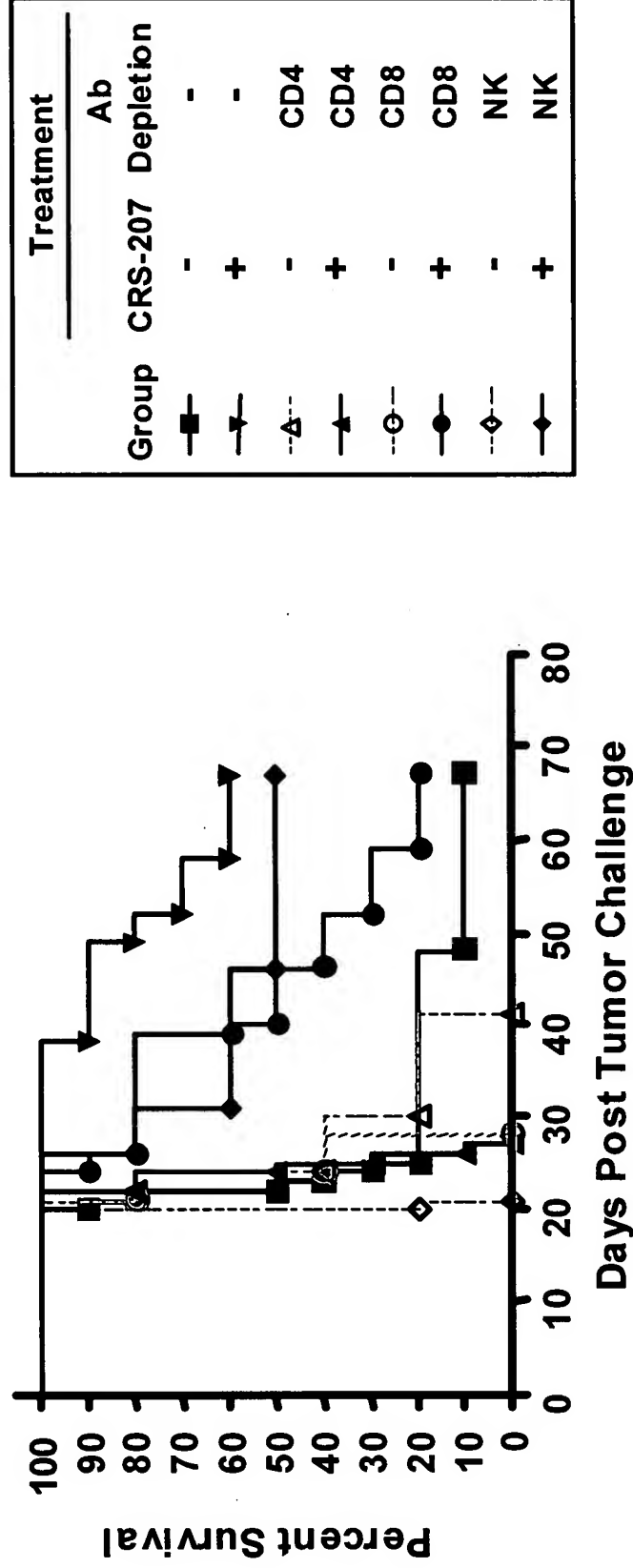
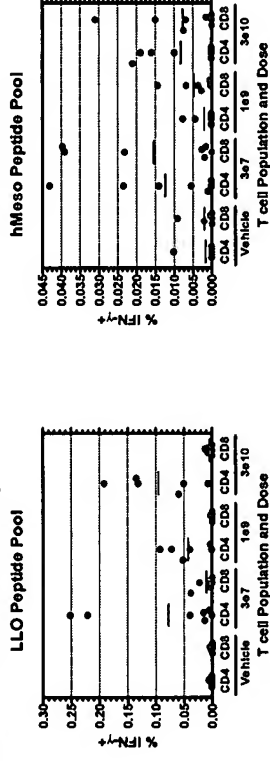


Figure 6. CRS-207 Immune correlates of survival. NK cell, CD4+ T cell, or CD8+ T cell in vivo depletion was initiated one week prior to tumor cell implantation followed by two additional treatments on Days 6 and 13 of the anti-AsialoGM, GK1.5, or 2.43 antibodies, respectively. Depletion of the respective lymphocyte population was confirmed by flow cytometry in separate cohorts of mice. A single treatment with 5×10^6 cfu of CRS-207 was initiated on Day 3 and mice were followed for survival. $n=10/\text{group}$.

CRS-207 Elicits Mesothelin-Specific Cellular Immunity in Cynomolgus Monkeys

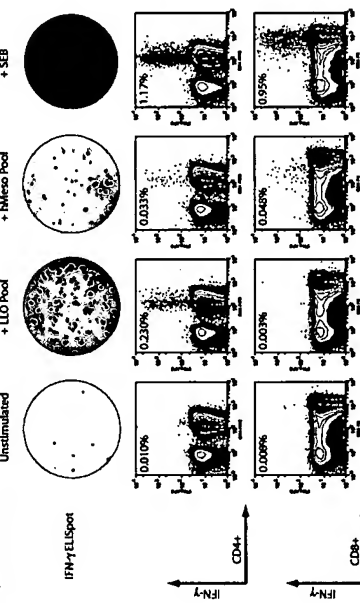
T cell responses to LLO and Mesothelin following prime-boost immunization with CRS-207

A. Responses of All Animals in Study



N = 4 animals / sex / dose group

B. Representative Animal



CRS-207 Dose: 3×10^7 cfu

Figure 7. CRS-207 Immunogenicity in Monkeys. Vaccine-induced Mesothelin- and *Lm* LLO- specific CD4+ and CD8+ T cell immunity was measured as a component of an ongoing Repeat-Dose GLP Toxicology Study with CRS-207 in Cynomolgus Monkeys. Shown are LLO- and Mesothelin-specific cellular immunity measured by ELISpot and ICS of PBMC from monkeys 6 days following a second intravenous (IV) immunization in which animals were given a prime-boost immunization regimen of 3×10^7 , 1×10^9 , or 3×10^{10} cfu of CRS-207, separated by 21 days. (A & B) T cell responses to *Lm* LLO and Mesothelin following prime-boost immunization with CRS-207. Intracellular cytokine staining: PBMC were restimulated for 6 hours with either the LLO peptide library or the human Mesothelin peptide library. Both libraries consisted of 15-mers overlapping by 11-AA spanning the entire protein. Restimulation took place in the presence of monensin and anti-CD28+anti-CD49d. Cells were surface stained with anti-CD3, anti-CD4, and anti-CD8. Following fixation and permeabilization, cells were stained with anti-IFN-γ. Samples were gated to include CD3+ events, then either CD4+ or CD8+ events individually for quantization of IFN-γ producing cells. ELISpot analysis of T cell responses: PBMC were seeded into plates coated with anti-IFN-γ capture antibody and the indicated stimulus added. 16 hours later, cells were washed from plate and bound cytokine was detected using anti-IFN-γ biotin following by streptavidin-Alkaline Phosphatase. (B) Representative ELISpot and ICS results from a Cynomolgus macaque with Mesothelin-specific CD4+ and CD8+ T cells. Percentage in upper left corner of cytometry plots represents the frequency of IFN-γ+ cells within either the CD4+ or CD8+ T cell population.

Ad Prime and CRS-207 Boost Immunization Induces Robust Mesothelin-Specific Cellular Immunity

HLA-A2 transgenic mice

A. Mesothelin 15 x 11 peptide library

B. HLA-A2 Meso₅₄₀₋₅₄₉ peptide

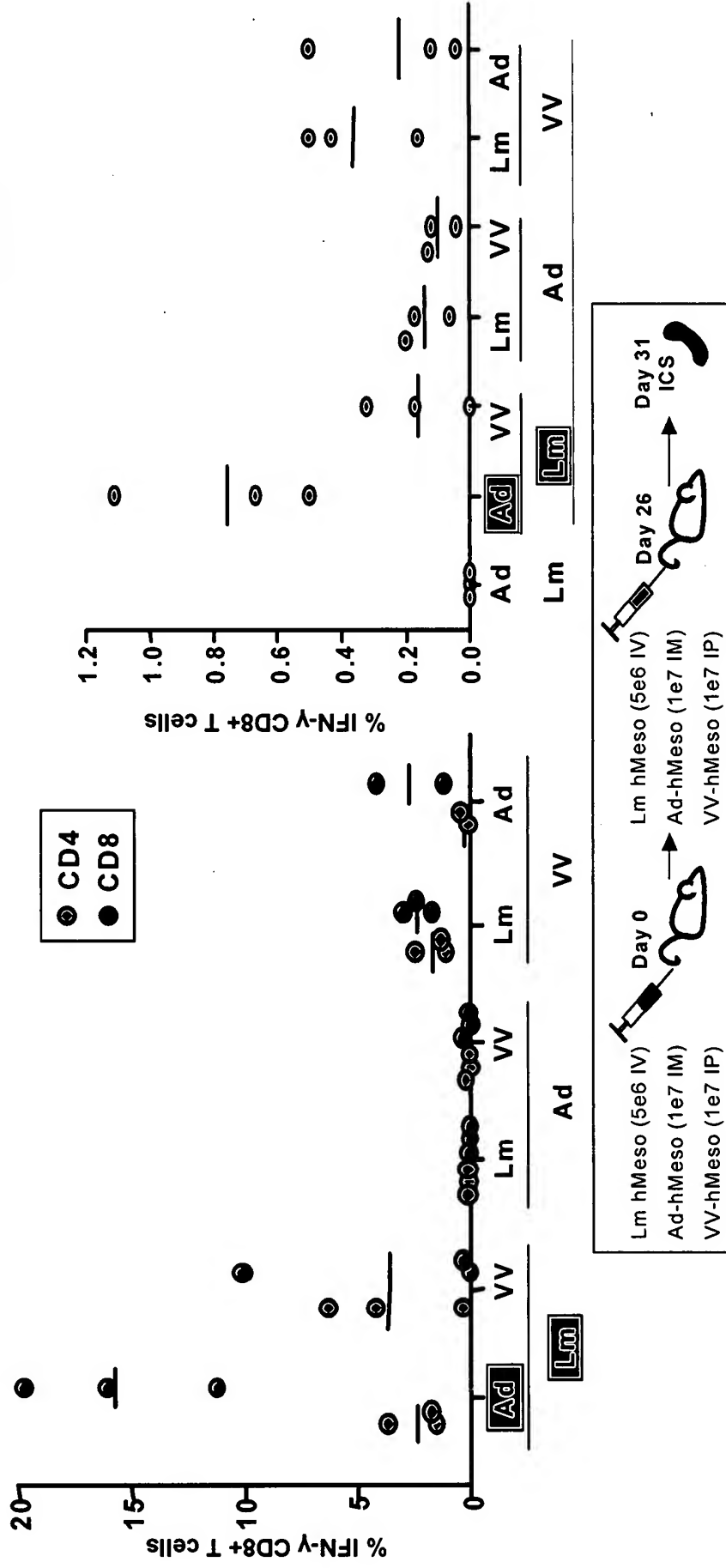
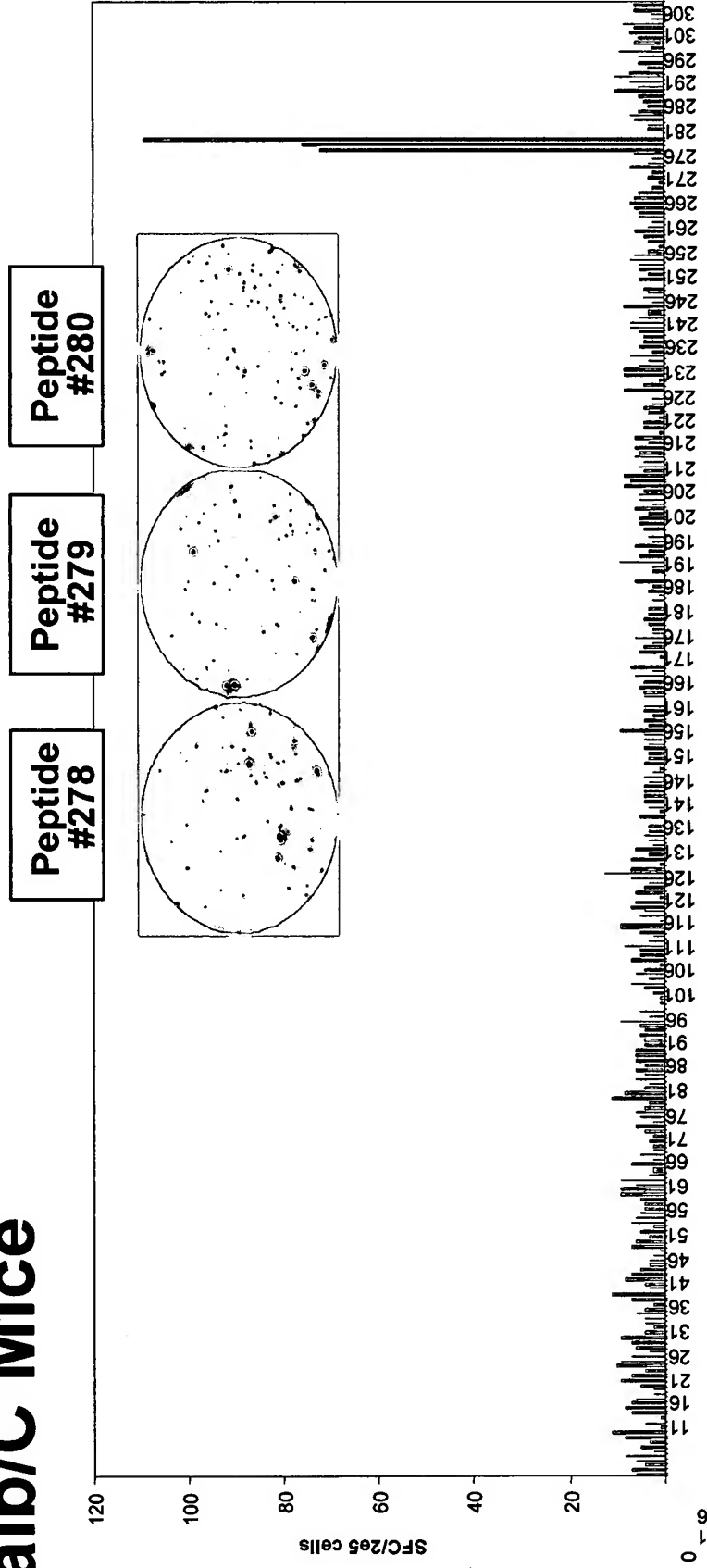


Figure 8. Ad prime and CRS-207 boost immunization regimen elicits robust Mesothelin-specific cellular immunity in HLA-A2 transgenic mice. Mice were vaccinated according to the regimen shown in the Figure. Splenic Mesothelin-specific cellular immunity was determined 5 days following the second vaccination by ICS, using a human Mesothelin peptide library described in Figure 7 legend (A), or the HLA-A2 restricted Meso₅₄₀₋₅₄₉ peptide (KLLGPHVEGL) (B), as stimulators.

Heterologous Prime-Boost Immunization Breaks Tolerance to Mouse Mesothelin in Balb/C Mice



Mouse Mesothelin Peptide Library

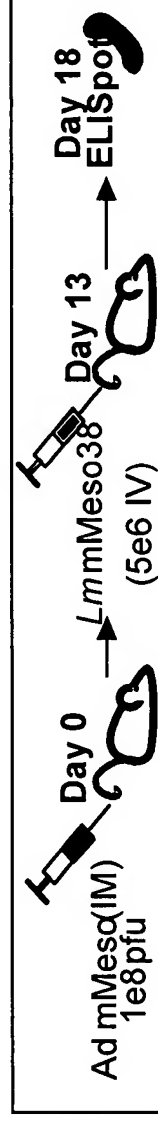


Figure 9. Prime-Boost [Immunization with Adenovirus and Listeria encoding mouse Mesothelin breaks tolerance against the endogenous antigen]. Balb/c mice were vaccinated according to the regimen shown in the Figure using Adenovirus and *Lm* vectors encoding mouse Mesothelin. Splenic Mesothelin-specific cellular immunity was determined 5 days following the second vaccination, using a mouse Mesothelin peptide library that was comprised of 12-mer peptides offset by 2 amino acids covering the entire length of the protein, as stimulators.

Summary

- Positive clinical outcomes correlated with Mesothelin-specific cellular immunity have been observed in patients with pancreatic carcinoma after vaccination with an irradiated allogeneic whole cell vaccine encoding GM-CSF (Jaffee et al, 2001)
- CRS-207 is a recombinant live-attenuated *L. monocytogenes* vaccine strain that encodes human Mesothelin
- CRS-207 elicits Mesothelin-specific cellular immunity in mice and in non-human primates, and therapeutic efficacy in tumor-bearing mice
- Prime and boost immunization with Adenovirus and *L. monocytogenes* vectors encoding mouse Mesothelin breaks tolerance against the endogenous antigen

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